CHARACTERIZATION OF FIMBRIAE OF *ACTINOMYCES NAESLUNDII* N16 USING MONOCLONAL AND POLYCLONAL ANTIBODIES

By

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TABLE OF CONTENTS

	page
ACKNOWLEDGMENTS	ii
ABSTRACT	v
INTRODUCTION	1
MATERIALS AND METHODS	8
Antigen Preparation	8
Cultures	8
Culture Conditions	9
Preparation of Crude Soluble Antigens	9
Preparation of Fimbriae for Purification	11
Sonication	11
French press shearing	12
Gel Filtration Chromatography	13
Treatments of Fimbriae by Physical/Chemical Means	14
Antibody Preparation	17
Immunization of Mice	17
Monoclonal Antibodies	17
Isotyping	18
Polyclonal Antibodies	18
DEAE Chromatography	19
Protein A-Sepharose Chromatography	20
Radiolabeling	20
Immunoaffinity Chromatography	20
Dissociation Experiment	21
Assays	23
Electron Microscopy	23
Hemagglutination	24
Coaggregation	25
Bacterial Agglutination	26
Radioimmunoassay	26
Indirect Enzyme Immunoassay	27
Enzyme Immunodot Assay	28
Immunodiffusion	28
Laurell Rocket Immunoelectrophoresis	29
Crossed Immunoelectrophoresis with Autoradiography	29
Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis	29
Immunoblot	30

RESULTS AND DISCUSSION..... 31

Monoclonal Antibodies..... 31
Evidence for the Presence of Both Types
of Fimbriae on A. naeslundii Serotype 3 Strains..... 35
Evidence for the Presence of Type 1 Fimbriae..... 35
Evidence for the Presence of Type 2 Fimbriae..... 43
Antigen Preparation: Results of Preliminary Experiments..... 46
Identification of N16 Type 1 and Type 2 Fimbriae
in Crude Antigen Extracts by XIEP-A..... 49
Purification of N16 Type 2 Fimbriae from a Crude Sonicate..... 51
Batch A..... 51
Batch B..... 54
Purification of N16 Fimbriae from the
French Press Supernatant..... 60
Effects of Various Physical and Chemical Treatments
on N16 Fimbriae..... 64
Effects of Temperature and Reduction on N16 Fimbriae..... 77
Assessment of the Purity
of Fimbrial Samples by SDS-PAGE-Immunoblot..... 83
Antigenic Relatedness of Actinomyces Fimbriae..... 90
Ouchterlony Analysis..... 90
XIEP-A..... 92
Bacterial Agglutination..... 106
Immunoblot Analysis..... 115

SUMMARY AND CONCLUSIONS..... 119

REFERENCES..... 127

BIOGRAPHICAL SKETCH..... 137

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

CHARACTERIZATION OF FIMBRIAE OF ACTINOMYCES NAESLUNDII N16 USING MONOCLONAL AND POLYCLONAL ANTIBODIES

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Two populations of fimbriae, which differ both in antigenicity and biological activity, have been identified on Actinomyces viscosus T14V cells. Although A. naeslundii serotype 1 isolates possess only one of these fimbrial populations (type 2 fimbriae), there was functional evidence to suggest that A. naeslundii serotype 3 strain N16 had both types of fimbriae. The purpose of this study was to characterize the fimbriae of A. naeslundii N16 immunologically by using both monoclonal and polyclonal antibodies.

Three monoclonal antibodies (MAbs) to N16 were produced; all three bound to N16 fimbriae as determined by immunoelectron microscopy. In a solid-phase radioimmunoassay MAb 3B5.A1 reacted with 100% of the A. naeslundii serotype 3 isolates tested, but it did not react with any heterologous isolates. Type 1 and type 2 fimbriae were detected in Lancefield extracts of N16 cells by crossed immunoelectrophoresis (XIEP) using rabbit antiserum against N16 whole cells. When ^{125}I-MAb 3B5.A1 was also incorporated into the gel, autoradiography indicated that MAb

3B5.A1 was specific for type 2 fimbriae. The N16 type 2 fimbriae were purified by gel filtration and immunoaffinity chromatography on a MAb 3B5.A1 column.

Polyclonal antisera specific for N16 type 1 or type 2 fimbriae were produced by immunizing rabbits with fimbrial immunoprecipitins excised from XIEP gels. These antisera were used to identify fimbrial bands on immunoblots of SDS-PAGE resolved N16 fimbrial antigens. Although N16 fimbriae could not be completely dissociated, type 1 fimbrial subunits at 65 kd, 57-60 kd, and a weaker doublet at 53-54 kd and type 2 subunits at 63 kd and a doublet at 39-40 kd were identified in fimbrial extracts reduced at 100^{o}C.

Fimbriae-specific polyclonal and monoclonal antibodies were used in various immunological assays to determine that (a) N16 type 1 fimbriae are not related antigenically to type 2 fimbriae, (b) each type of fimbriae has epitopes that are present on the corresponding fimbriae of certain heterologous strains, and (c) MAb 3B5.A1 recognizes a serotype-specific epitope residing on the type 2 fimbriae of A. naeslundii serotype 3 strains.

INTRODUCTION

Many structures found on the cell surfaces of microorganisms exhibit properties that enable microorganisms to cause disease. These properties include (a) adherence to host surfaces and other microbes, (b) evasion of host defenses, and (c) destruction of host tissues. In addition, surface molecules carry antigenic determinants that form the basis of many serological and immunological identification procedures. The fimbriae of certain species of Actinomyces are one example of surface components that not only mediate adherence both to host surfaces and to other microbes but also possess important antigenic determinants.

Actinomyces are gram-positive, nonacidfast, nonsporeforming, nonmotile bacteria that are highly variable in morphology, most characteristically diphtheroidal, or filamentous and branched. They ferment carbohydrate with production of acid but no gas, and except for A. viscosus, all species are catalase-negative. Their cell walls do not contain diaminopimelic acid, a characteristic that differentiates them from Arachnia propionica, a morphologically similar organism. Their natural habitat is the oral cavity of man and other animals (96). All Actinomyces species, as well as Arachnia propionica, are potential agents of actinomycosis (45).

Classical actinomycosis is a chronic granulomatous disease characterized by the formation of abscesses and draining sinuses. It is an endogenously acquired infection that can involve the soft tissue

and bone in any area of the body. Clinically, actinomycosis is usually categorized as cervicofacial, thoracic, or abdominal. Of these categories, cervicofacial actinomycosis is the most common, and Actinomyces israelii is the most important etiological agent of actinomycosis in humans. However, the extraoral infections of classical actinomycosis are not as prevalent as intraoral periodontal infections, and it has been suggested that the clinical classification scheme be broadened to include a periodontal category (56).

During the last twenty-five years, most of the research efforts dealing with Actinomyces species have been directed toward trying to delineate the role that Actinomyces species play in the formation of dental plaque and the development of periodontal disease. In the early 1960s A. viscosus was found to be the etiologic agent of a transmissible periodontal disease in Syrian hamsters (59,65). That discovery initiated an explosion of research activity focused on the pathogenicity of Actinomyces species in the oral cavity of man and other animals because periodontal disease, unlike classical actinomycosis, was and still is a major public health problem. Virtually all humans and many animals accumulate dental plaque on their teeth, and the formation of plaque may be followed by the development of both caries and periodontal disease (49).

Periodontal disease is a collective term for a variety of chronic inflammatory diseases of the structures that support the teeth. Periodontal disease can range from mild inflammation of the marginal gingiva (gingivitis) to severe forms of periodontitis, in which extensive destruction of soft tissues and resorption of alveolar bone can result in loss of the teeth. Although the microbial etiology and

pathology of the different types or stages of periodontal disease may vary (98), the formation of dental plaque is the common first step in the development of periodontal disease (49). However, successful colonization of the tooth surface or other surfaces of the oral cavity depends upon the ability of oral microbes to anchor themselves, either directly or indirectly, to these surfaces to avoid being swept away by the flow of saliva (47,104). Thus, an understanding of the mechanisms by which microbes attach to surfaces in the oral cavity and to other microbes is crucial to understanding the sequence of events leading to periodontal disease.

Studies with experimental animals indicated that both A. viscosus and A. naeslundii could form dental plaque and initiate the pathological changes associated with periodontal disease (55,58,59,60,97). However, most of the attention has been focused on A. viscosus, particularly in studies on mechanisms of attachment to oral surfaces and other bacteria (6,13,14,16,19,21,22,27,48,72,81,82, 92,106,107) and mechanisms for tissue destruction (1,8,9,10,39,40,51, 53,74,78,94).

There were two factors that contributed to the placing of more emphasis on A. viscosus than A. naeslundii as a subject for periodontal research. One was the recognition that these two species tended to be distributed differently in the human oral cavity in that A. viscosus preferentially colonized the teeth, whereas A. naeslundii was associated with mucosal epithelial surfaces (34,35). The second was the existence of the T14V-T14AV model system for comparative studies of the properties that enable T14V to be virulent (i.e., cause periodontal disease) in experimental animals and T14AV to be avirulent

(6,51). Although it was soon determined that the virulence-associated differences in the antigens of T14V and T14AV were quantitative rather than qualitative (19,22,90), T14V became the focus of intensive study because it was found to have two different adherence-related properties. These were the ability to coaggregate with Streptococcus sanguis via a lactose-inhibitable lectin (82) and the ability to adsorb well to saliva-coated hydroxyapatite (24). A. naeslundii, on the other hand, coaggregated streptococci (36) but did not bind well to saliva-coated hydroxyapatite (24). Some of these early studies established an association between the presence of fibrils or fimbriae on the surface of A. viscosus cells and adherence-related functions (19,22,82).

Fimbriae are proteinaceous surface appendages that are found on many gram-negative and gram-positive bacteria and that often mediate attachment to host surfaces. Fimbriae from five strains of A. viscosus and two strains of A. naeslundii have been isolated and characterized as to their amino acid compositions (21,76,77,106). Polar uncharged and nonpolar amino acids together made up 62-74% of the total amino acids, whereas basic amino acids accounted for only 10-17% of the total; aspartic and glutamic acid generally comprised 20-24% of the total amino acids (76). The minimum molecular weight of a fimbrial subunit as calculated from amino acid data was determined to be approximately 25 kd for T14V (type 1) fimbriae (106) or 30 kd for A. viscosus WVU627 fimbriae (77). However, a method for completely dissociating Actinomyces fimbriae into subunits has not been discovered (12).

Over the last decade, the fimbriae of A. viscosus and A. naeslundii have been the subject of intensive study. A great deal of progress has been made in identifying the adherence-related functions of the fimbriae and defining the molecular basis of the interactions of certain Actinomyces cells with other microorganisms and surfaces within the oral cavity. Nearly all of the studies have focused on only two strains, A. viscosus serotype 2 strain T14V and A. naeslundii serotype 1 strain WVU45, and spontaneously occurring mutants derived from these two strains. Two populations of Actinomyces fimbriae, which differ both in antigenicity and biological activity, have been identified (20,84). Type 1 fimbriae mediate adherence to saliva-coated hydroxyapatite in vitro and to the salivary pellicle on teeth in vivo (23). Type 2 fimbriae via their lactose-inhibitable lectin activity mediate adherence to other oral bacteria and mammalian cells (11,12,15,44,68,80,93,103). The functional and molecular properties of the fimbriae from these two strains have been recently reviewed (84).

Both types of fimbriae are present on T14V and other serotype 2 isolates of A. viscosus, whereas WVU45 and other A. naeslundii serotype 1 isolates possess only type 2 fimbriae (20). In 1974 Jordan et al. (57) described a new serotype of A. naeslundii based on their studies of strain N16 and 20 similar isolates. These strains had been isolated from cervical plaque in a sampling of 59 Down's syndrome patients who had moderate to severe periodontal disease (64). In studies with gnotobiotic rats and hamsters, Jordan and co-workers have shown that N16 can cause heavy plaque deposits and severe periodontal pathology, including alveolar bone loss, root surface caries,and even

enamel caries in some animals (57,60). Strain N16 and isolates that are biochemically and serologically identical to it have been designated A. naeslundii serotype 3 (46).

There is both functional and immunological evidence to suggest that A. naeslundii serotype 3 strain N16 has both types of fimbriae. The N16 isolate exhibits lactose-reversible coaggregation with Streptococcus sanguis 34 (18). It produces its own neuraminidase and agglutinates human erythrocytes in a reaction that is reversed by lactose (29). It possesses numerous surface fibrils or fimbriae and adsorbs to saliva-treated hydroxyapatite (SHA) in vitro as avidly as T14V (26). In fact, Clark et al. (25) demonstrated that on average A. naeslundii serotype 3 adsorbed better to SHA than any other species or serotype of Actinomyces tested. Strain N16 agglutinated with MAbs against T14V type 2 fimbriae (14). It reacted with rabbit IgG against T14V type 1 fimbriae in a dot enzyme immunoassay (28), and its adsorption to SHA was partially inhibited by the antibody (23,28).

Although most of our knowledge of Actinomyces fimbriae has been based on studies of A. viscosus serotype 2 T14V and A. naeslundii serotype 1 WVU45, A. naeslundii serotype 3 was the Actinomyces species most frequently isolated from subgingival dental plaque from sites with moderate, severe, or juvenile periodontitis (87), and in another study A. naeslundii serotype 3 was considered to be one of the most likely etiological agents of gingivitis (86). Thus, A. naeslundii serotype 3 strain N16 appears to be a logical choice for a study of surface antigens of a periodontopathic actinomycete.

The goals of this study were to (a) produce monoclonal antibodies against surface antigens of A. naeslundii serotype 3 strain N16, (b) select at least one MAb that exhibited serotype specificity, and (c) use the MAb as a tool to isolate and characterize the surface component carrying the serotype-specific epitope.

MATERIALS AND METHODS

Antigen Preparation

Cultures

A. naeslundii N16 (WVU820), WVU1267, WVU1468, WVU1527, and WVU1528 were obtained from M. A. Gerencser, West Virginia University. W1629, W2273, and W2821 were obtained from the Centers for Disease Control, Atlanta, GA. UF92 and UF524 were fresh clinical isolates provided by J. E. Beem, University of Florida, Gainesville, FL. The identity of each of these ten isolates was confirmed by biochemical tests and by direct staining with serotype-specific fluorescent antibody reagents; all ten isolates were designated *A. naeslundii* serotype 3. Approximately 50 additional isolates, including representatives of all the recognized serotypes of *A. naeslundii*, *A. viscosus*, *A. israelii*, *A. odontolyticus*, *Arachnia propionica*, *Rothia dentocariosa*, and *Bacterionema matruchotii* were obtained from culture collections at the Centers for Disease Control for use as heterologous organisms in evaluating the specificity of monoclonal antibodies. Other strains used in this study were kindly provided by P. E. Kolenbrander, National Institute of Dental Research, Bethesda, MD, and by W. B. Clark and P. J. Crowley, University of Florida. Stock cultures of all isolates used in this study were maintained by lyophilization in skim milk and by storage at -20°C in Trypticase soy broth (TSB; BBL Microbiology Systems, Cockeysville, MD) containing 20% (v/v) glycerol.

Culture Conditions

Cells for all procedures were obtained by culturing the isolates in TSB supplemented with 2.5 g of K_2HPO_4 per liter in flasks that could be chemically sealed to provide the atmospheric environment appropriate for each isolate. After inoculation of a TSB-containing flask with an actively growing TSB culture (2% v/v), the flask was plugged with a rubber stopper through which an open-ended screw-capped tube filled with cotton had been inserted (7). Prior to tightening of the screwcap, the cotton was saturated with equal volumes of 10% Na_2CO_3 and 1 M KH_2PO_4 to generate an aerobic + CO_2 environment or with 10% Na_2CO_3 and pyrogallol solution to generate an anaerobic + CO_2 environment (45). The cultures were incubated without shaking at 37°C. Cells were harvested from cultures in mid exponential to early stationary phase by centrifugation (10,000 x g, 10 min), washed twice, and stored in buffer at 4°C. The buffers used for washing and making cell suspensions varied depending on the procedure for which the cells were intended; they are noted in each specific method.

Preparation of Crude Soluble Antigens

Several different methods were used to obtain crude soluble antigens from whole cells.

Ammonium sulfate fractionation of French pressure cell supernatants. N16 cells harvested from 8.5 l of 36 h TSB cultures were suspended in 0.05 M phosphate buffer, pH 7.2, to form a thick paste; then the cell suspension was passed once through a French pressure cell (Aminco Model J4-3337, American Instrument Co., Silver Springs, MD) at 10,000 psi to remove the fimbriae (106). The

pressed cell suspension was centrifuged (48,300 x g, 20 min) to pellet intact cells and cell walls; then the crude supernatant was ultracentrifuged at 160,000 x g for 24 h to pellet the fimbriae. The pellet was partially resuspended in 0.1 M Tris-HCl, pH 7.5, by sonicating for 1 min at full power with a Kontes microultrasonic cell disruptor (Kontes, Vineland, NJ). After centrifugation at 23,700 x g for 10 min, the fimbriae-containing supernatant was processed by ammonium sulfate fractionation to obtain precipitates at 10, 20, 30, 40, and 50% (w/v) $(NH_4)_2SO_4$. The precipitates were collected by centrifugation (30,900 x g, 15 min), dissolved in and dialyzed against deionized water, and lyophilized. Lyophilized antigens were dissolved in 0.5% Triton X-100 by adding 100 µl to 2 mg dry weight and sonicating for 10 sec. The lyophilized material was highly resistant to solubilization, so lyophilization was not used on subsequent batches of fimbriae.

Continuous flow sonication. N16 cells (10% packed cells by volume) in Tris-buffered saline (TBS; 0.025 M Tris-HCl, 0.15 M NaCl, 10^{-4} M $CaCl_2$, 10^{-4} M $MgCl_2$, and 0.02% NaN_3, pH 7.6) were passed at a flow rate of 5 ml/min three times through a continuous flow sonifier cell disruptor (Heat Systems Ultrasonics, Inc., Farmingdale, NY) set at 100 W (14).

Virtis homogenization. A suspension of N16 cells in buffer was agitated twice at 23,000 rpm for 5 min in a Virtis Model 45 homogenizer (The VirTis Company, Inc., Gardiner, NY) (77).

Extraction with 8 M lithium chloride. N16 cells were suspended in 8 M LiCl, pH 7.0, (10% packed cells by volume) and stirred continuously for 1 h at 25°C (82).

Lancefield extraction. N16 cells were suspended in 0.04 N HCl in saline (5 ml per g wet weight of cells); the suspension was heated for 15 min in a boiling water bath, cooled, and neutralized (90).

For the last four methods described above, the cells and supernatants were separated by centrifugation (10,000 x g, 10 min), and the supernatants were concentrated by ultrafiltration (PM10 membrane; Amicon Corp., Danvers, MA). The protein content of each sample was determined by the Bio-Rad Protein Assay, a Coomassie blue dye binding assay (3) (Bio-Rad Laboratories, Richmond, CA). Total carbohydrate was estimated by the phenol-sulfuric acid procedure (33).

Preparation of Fimbriae for Purification

Sonication

Batch A. N16 cells were harvested from 12.5 l of 36 h TSB cultures, washed, and resuspended in TBS to a total volume of 240 ml (10-20% v/v). Fimbriae were removed by sonicating 30 ml aliquots in 15 sec bursts at 100 W. After 0.5, 1, 2, 3, 4, 5, and 6 min of sonication, cells and supernatants were separated by centrifugation (10,000 x g, 10 min). The cells were resuspended in fresh buffer, and a few drops of the suspension were set aside for subsequent examination by hemagglutination (HA) and electron microscopy (EM) before the next cycle of sonication was started. All the supernatants were pooled and centrifuged (27,000 x g, 30 min) to remove particulates. The crude 6 min sonicate was concentrated to 10 ml by ultrafiltration (Amicon PM10 membrane) and by addition of dry Sephadex G-25 prior to chromatography on Bio-Gel A-5m.

Batch B. N16 cells were harvested from 27.5 l of 24-29 h TSB cultures, washed, and resuspended in TBS to a total volume of 750 ml

(10-20% v/v). Fimbriae were removed from 720 ml of cell suspension by sonicating 30 ml aliquots in 30 sec bursts at 100 W. After sonication for a total of 2 min, the cell suspensions were centrifuged at 10,000 x g for 10 min to separate intact cells from supernatants. The sonicated cells were resuspended in fresh buffer for examination by HA. The supernatant or crude 2 min sonicate was further clarified by centrifugation (27,000 x g, 30 min), then concentrated by ultrafiltration (Amicon PM10 membrane) to 180 ml. The Amicon concentrated sonicate was mixed with an equal volume of saturated $(NH_4)_2SO_4$ to give 50% saturation. After incubating at 4^oC overnight, the precipitate was collected by centrifugation (10,000 x g, 30 min), dissolved in water, and re-precipitated with $(NH_4)_2SO_4$ at 35% final saturation. The precipitate from the 35% $(NH_4)_2SO_4$ saturation was collected by centrifugation, resuspended in water, and dialyzed against TBS. This was the starting material for gel filtration chromatography.

French press shearing

N16 cells were harvested from 30 l of 16-24 h TSB cultures, washed once in TBS, and resuspended in TBS (5 ml of TBS per l of culture harvested). The cell suspension was passed once through a French pressure cell (Model J4-3337; American Instrument Company, Silver Spring, MD) at 10,000 psi.

The amount of lysis caused by the French press was estimated to be 1.2%, as determined by the following equation:

$$\% \text{ lysis} = \frac{(A260/A280 \text{ of untreated}) - (A260/A280 \text{ of treated})}{A_{260}/A_{280} \text{ of untreated}} \times 100$$

Intact cells were removed from the pressed cell suspension by centrifugation at 10,000 x g for 10 min. The French press supernatant was clarified further by centrifugation at 48,300 x g for 20 min; the clear supernatant was stored at -80°C until processed.

Gel Filtration Chromatography

Columns were packed with Bio-Gel A-5m (exclusion limit 5 x 10^6 daltons for globular proteins; Bio-Rad Laboratories). The void and total volumes were determined with blue dextran 2000 and potassium ferricyanide, respectively. Crude sonicates were applied to the columns and eluted with TBS. A Marriotte flask was used to maintain a constant operating pressure. For all chromatographic techniques, effluents and eluates were monitored by A_{280} with a Uvicord II UV analyzer and recorder, and fractions were collected with an Ultrorac fraction collector (LKB Instruments Inc., Rockville, MD). The Batch A sonicate was run on a 12 x 750 mm column packed to a bed volume of 77.5 ml; the flow rate was 8 ml/h, and 3 ml fractions were collected. The Batch B sonicate, a 35% saturated $(NH_4)_2SO_4$ fraction, was applied in several aliquots (20 ml each at 3.9 mg protein per ml) to a 25 x 1000 mm column packed to a bed volume of 410 ml; the flow rate was 25 ml/h, and 5 ml fractions were collected. Column fractions were assayed for (a) total protein by the Bio-Rad Protein Assay with bovine albumin as the standard, (b) total carbohydrate by the phenol-sulfuric acid method with glucose as the standard, and (c) fimbriae reactive with MAb 3B5.A1 by an enzyme immunodot assay. Some fractions were also examined by SDS-PAGE.

Treatments of Fimbriae by Physical/Chemical Means

Acetone precipitation. Five volumes of cold acetone were added to the sample. After having been mixed thoroughly, the sample was incubated at -20°C for at least 10 min. Precipitates were collected by centrifugation (10,000 x g, 5 min) and dried by evaporation under vacuum. Precipitates were reconstituted in an appropriate buffer.

Magnesium chloride precipitation. Four parts of 1 M $MgCl_2$ were mixed with one part sample to obtain a final concentration of 0.1 M $MgCl_2$. The sample was incubated at 4°C for 24 h, then examined for evidence of precipitation.

Freon extraction. Equal volumes of sample and Freon 113 (trichlorotrifluoroethane) were mixed thoroughly by vortexing. After centrifugation (2000 x g, 30 min), the upper aqueous phase was collected.

Heat. Samples were placed in a waterbath at 37°C for 60 min, at 65°C for 30 min, or at 100°C for 5 min or 60 min.

Sonication. Samples in capped microfuge tubes were placed in an ice slurry in a cup horn sonicator (Heat Systems-Ultrasonics, Inc., Plainview, NY) and sonicated continuously at full power for 1, 5, or 10 minutes.

Urea. An acetone precipitate of the sample was dissolved in a volume of 8 M urea in 0.05 M Tris-HCl, pH 8.0, equal to the original volume of sample; then it was incubated at 37°C for 1 h.

Guanidine hydrochloride. An acetone precipitate of the sample was dissolved in saturated (8.6 M) guanidine HCl at a final concentration of 1 ml of guanidine HCl per mg total protein. After incubation at 37°C for 1 h, the sample was diluted with and dialyzed against 10 mM EDTA, 0.05 M Tris-HCl, 0.15 M NaCl, 0.1% NaN_3, pH 7.5.

Acid. One aliquot was treated by Lancefield extraction; it was adjusted to 0.04 N HCl in saline and heated at 100°C for 15 min. Two others were adjusted to 0.1 N HCl and heated at 100°C for 5 min or at 37°C for 1 h. All were neutralized by addition of NaOH after treatment.

Base. The sample was adjusted to 0.1 N NaOH and incubated at 37°C for 1 h; then it was neutralized with HCl.

Periodate oxidation. The sample was adjusted to 0.1 M sodium metaperiodate in 0.05 M acetate buffer, pH 4.5, and incubated in the dark at 4°C for 24 h; then ethylene glycol was added to 0.3 M final concentration to consume excess periodate.

Enzymatic digestions. Solutions of lysozyme (Cat. No. 36-324, Miles Laboratories, Naperville, IL), mutanolysin (M-3765, Sigma Chemical Co., St. Louis, MO), papain (Sigma P-4762), and Pronase (Cat. No. 537088, Calbiochem, San Diego, CA) were prepared in 0.1 M sodium phosphate, pH 6.2. Solutions of α-chymotrypsin, trypsin (Sigma T-8253, Type III), Staphylococcus aureus V8 protease (Sigma P-8400, Type XVII), and proteinase K (Sigma P-0390, Type XI) were prepared in 0.05 M Tris-HCl, pH 8.0. Acetone precipitates of crude N16 French press supernatants were dissolved in 0.1 M phosphate-buffered saline, pH 6.2, or 0.05 M Tris-buffered saline, pH 8.0, depending on the enzyme to be used.

Antigen samples containing 25 μg of total protein were incubated (37°C, 30 min) with serial dilutions of lysozyme ranging from 0.1 μg to an upper limit of 7 μg (175 units) of lysozyme per sample. Likewise, samples were treated with mutanolysin in amounts ranging from 0.3 Units to 17.5 Units. To ensure that the lysozyme and

mutanolysin were active, a suspension of A. naeslundii PK19 cells was treated with each enzyme, and muramidase activity was detected as a decrease in turbidity (A_{600}) of the cell suspension.

Samples that were to be treated with proteases were heated at 100°C for 2 min in the presence of 0.5% SDS prior to the addition of enzyme solutions in order to make the proteins more susceptible to digestion and to inactivate any endogenous proteases that might be present in the crude antigen sample. Samples containing 500 µg of total protein were mixed with 50 µg of enzyme (chymotrypsin, trypsin, papain, Pronase, proteinase K, or V8 protease) for a 10:1 ratio of protein:enzyme, and the appropriate buffer was added to bring the final enzyme concentration to 100 µg per ml. The protease-treated samples were incubated in a 37°C waterbath for 1 h.

At the end of the incubation period each sample was prepared immediately for SDS-PAGE and stored at -80°C until it could be evaluated.

Aliquots of bovine serum albumin were treated exactly like the N16 samples as a positive control for activity of the proteases and for detection of protease contamination in the muramidases. Samples that received buffer in place of enzyme served as untreated controls. Samples containing enzyme only were also used as controls.

Treated samples were subjected to SDS-PAGE; the SDS-PAGE resolved proteins were transferred to nitrocellulose. These blots were reacted with either R10 rabbit IgG against N16 type 1 fimbriae or R2P rabbit IgG against N16 type 2 fimbriae in an indirect EIA to visualize immunoreactive fimbrial bands.

Antibody Production

Immunization of Mice

The cell suspensions for immunizations were formalin-killed whole cells suspended in phosphate-buffered saline (PBS; 0.01 M phosphate, 0.85% NaCl, pH 7.4) containing 0.3% formalin. Cell aggregates were dispersed by sonication, and the turbidity of each cell suspension was adjusted to a No. 8 McFarland Standard. To obtain antigen-primed spleen cells for fusions, 10-week old female BALB/c mice were immunized with A. naeslundii N16 cells. Two mice that received 0.1 ml intravenously on days 1, 8, and 16 were sacrificed on day 20 for fusion of their spleen cells with X63Ag8.653 myeloma cells. Two additional mice received 0.1 ml mixed with an equal volume of Freund's complete adjuvant intraperitoneally on day 1 and 0.1 ml without adjuvant intravenously on day 19 and were sacrificed on day 22 for a fusion with P3X63Ag8 cells.

Monoclonal Antibodies

Hybridomas were produced, using the method of Simrell and Klein (95), by fusing 10^8 spleen cells from N16-immunized mice with 10^7 myeloma cells in the presence of 50% polyethylene glycol-1000. For one fusion myeloma cell line P3X63Ag8, which secretes immunoglobulin molecules with gamma-1 heavy chains and kappa light chains (66), was used. For another fusion, the Kearney myeloma cell line X63Ag8.653, which does not express any immunoglobulin chains (63), was used. Hybrid cells were selected by growth in Dulbecco's modified Eagle's medium (DMEM) containing hypoxanthine, aminopterin, and thymidine (HAT medium). Culture supernatants were screened for specific antibody by solid-phase radioimmunoassay (RIA). Hybridomas secreting antibody to

N16 were cloned by limiting dilution by seeding a 96-well tissue culture plate at a density of one-half cell per well; each well contained 5 x 10^4 mouse peritoneal exudate cells as a feeder layer. Three different clones were selected for further evaluation. They were propagated in tissue culture and as ascites tumors in female BALB/c mice primed by intraperitoneal injection of 0.5 ml pristane (2,6,10,14-tetramethylpentadecane; Aldrich Chemical Company, Inc., Milwaukee, WI). Culture supernatants and sera or ascites fluids from hybridoma-bearing mice were stored at -20°C. Hybridoma cells in DMEM containing 30% fetal calf serum and 10% dimethylsulfoxide were stored in liquid nitrogen.

Isotyping

The class and subclass of immunoglobulin secreted by each hybridoma were determined initially by immunodiffusion in which subclass-specific anti-heavy chain antisera (Meloy Laboratories, Inc. Springfield, VA) were reacted with the hybridoma culture supernatants. These results were confirmed subsequently by testing hybridoma culture supernatants or ascites fluids with an enzyme immunoassay isotyping kit (Mouse Typer, Bio-Rad Laboratories).

Polyclonal Antibodies

Polyclonal antisera monospecific for type 1 and type 2 fimbriae of A. naeslundii N16 were produced in female New Zealand white rabbits by immunization with immunoprecipitins cut from crossed immunoelectrophoresis (XIEP) gels (71). Type 1 and type 2 fimbriae from partially purified samples of N16 fimbriae were separated and precipitated by XIEP versus rabbit antiserum R29 raised against N16 whole cells. Sections of gel containing the immunoprecipitate were

cut from several XIEP patterns. The appropriate segments (a minimum total of 10 cm per immunogen) were pooled, washed exhaustively with saline to remove excess soluble reactants, and solubilized in 1 ml of 6 M KI. One-fourth of the immunogen was mixed with an equal volume of complete Freund's adjuvant and injected subcutaneously into multiple sites. The remainder of the immunogen was emulsified with an equal volume of incomplete Freund's adjuvant and administered in 3 subcutaneous injections at approximately weeks 3, 5, and 7. Three rabbits received type 1 fimbrial immunoarcs, and three were immunized with type 2 fimbrial immunoarcs. Antibody responses were monitored by microtiter plate EIA, and additional injections of immunogen were given as needed to boost or maintain antibody titers. The rabbits were bled periodically from the central artery of the ear throughout the schedule and were exsanguinated by cardiac puncture 3-9 months after the first injection.

R10 against N16 type 1 fimbriae and R2P against N16 type 2 fimbriae were the two rabbit antisera used most often in this study. J. O. Cisar, National Institute for Dental Research, kindly provided the following samples of monospecific rabbit IgG: R59 against A. viscosus T14V type 1 fimbriae, R55 against T14V type 2 fimbriae, and R70 against A. naeslundii WVU45 type 2 fimbriae. Rabbit antiserum raised against immunoarcs of Histoplasma capsulatum was kindly provided by P. Standard, Division of Mycotic Diseases, Centers for Disease Control, for use as a negative control.

DEAE Chromatography

IgG was purified from rabbit antisera or hybridoma culture supernatants by chromatography on DEAE Bio-Gel A (Bio-Rad

Laboratories). Antisera and supernatants were precipitated with $(NH_4)_2SO_4$ at 50% saturation. The precipitates were dissolved and equilibrated with 0.01 M phosphate buffer, pH 7.8. Samples were applied to DEAE Bio-Gel A columns, and IgG was eluted with equilibrating buffer. Purified IgG was concentrated by ultrafiltration and stored at 4°C with 0.1% NaN_3 as preservative.

Protein A-Sepharose Chromatography

Protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) was packed in a column with flow adaptors. Hybridoma culture supernatants or rabbit antisera were precipitated with ammonium sulfate at 50% saturation. The precipitates were dissolved in water and equilibrated against starting buffer. Samples were applied and the unbound fraction eluted in 0.05 M Tris-HCl, 0.25 M NaCl, 0.1% NaN_3, pH 8. IgG was eluted with 7 M urea in 0.05 M Tris-HCl, pH 8.

Radiolabeling

Purified immunoglobulins in PBS and $Na^{125}I$ (5 μCi per μg protein) were placed in glass tubes coated with IODO-GEN (1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril; Pierce Chemical Co., Rockford, IL). The samples were incubated for 10 min at 25°C, and then removed from the reaction vessel to terminate the iodination. ^{125}I-labeled Igs were separated from free $Na^{125}I$ by gel filtration on PD-10 columns (Pharmacia) equilibrated with PBS containing 1% bovine serum albumin and 0.1% NaN_3. All ^{125}I-labeled samples contained 90-100% TCA-precipitable counts per minute.

Immunoaffinity Chromatography

MAb 3B5.A1, purified from hybridoma culture supernatants by protein A-Sepharose chromatography, was coupled to Affi-Gel 10

(Bio-Rad) and packed in a K16/20 column with flow adaptors (Pharmacia); bed volume was 9.5 ml. Samples were applied by continuously cycling them through the column in an ascending direction at a flow rate of 10 ml/h. Unbound and bound fractions were eluted at a flow rate of 40 ml/h in an ascending or descending direction, respectively. The unbound fraction was eluted with 0.05 M Tris-HCl, 0.25 M NaCl, 0.1% NaN_3, pH 8. The bound fraction was eluted with 7 M urea in 0.05 M Tris-HCl, pH 8.

Rabbit IgG, 250 mg in 0.1 M HEPES, pH 7.5, purified by DEAE and protein A-Sepharose chromatography from antisera monospecific for N16 type 1 or type 2 fimbriae, was coupled to 50 ml of Affi-Gel 10 and packed in a K26/40 column with flow adaptors. The coupling efficiency for each gel was >90%. Initially samples were eluted as described for the MAb immunoaffinity column; however, on the basis of results of a dissociation experiment, 6 M NaSCN was substituted for 7 M urea in the buffer for elution of the bound fimbriae.

Dissociation Experiment

For protein A-Sepharose chromatography and initial experiments with immunoaffinity chromatography, 7 M urea was used as the dissociating buffer for release of bound molecules from the ligand. However, to experimentally determine the most effective dissociating buffer to use with each immunoaffinity column, various dissociating buffers were evaluated in a microtiter plate EIA for their efficacy in releasing antibody bound to antigen.

The wells of a 96-well Immulon 2 plate (Dynatech Laboratories, Inc., Alexandria, VA) were coated with a crude fimbriae-containing sonicate of N16 cells. Aliquots of the antibody samples used to

prepare the three immunoaffinity columns, i.e., MAb 3B5.A1, rabbit IgG anti-N16 type 1 fimbriae, and rabbit IgG anti-N16 type 2 fimbriae, were the antibody samples tested in the dissociating experiment; the optimal dilution of each antibody had been determined by indirect EIA versus the fimbriae-coated plates. An indirect EIA was performed as described below, except that prior to the addition of the enzyme conjugate, each well received 200 ul of a dissociating or control buffer for 1 h at 25°C. Buffers were evaluated in triplicate, and the average A_{490} of the triplicate samples was used in the following formula for measuring the efficacy of each buffer in dissociating the antigen-antibody bonds:

$$\% \text{ release of Ab from fimbriae} = 1 - \frac{(A490 \text{ with dissociating buffer})}{(A490 \text{ with control buffer})} \times 100$$

The control buffer was the buffer normally used to elute the unbound fraction during immunoaffinity chromatography, i.e., 0.05 M Tris-HCl, 0.25 M NaCl, 0.1% NaN_3, pH 7.5 at 25°C.

In order to rule out the possibility that the dissociating buffers themselves were removing the fimbriae from the plate or adversely affecting the fimbriae in some other manner rather than merely dissociating the antibody, an indirect EIA was performed on a fimbriae-coated plate that was first incubated with dissociating buffers at maximum concentration overnight at ambient temperature. If the dissociating buffers were releasing fimbriae from the plates or irreversibly denaturing fimbrial epitopes, the A_{490} of wells that were incubated with dissociating buffers would be lower than that of controls.

Assays

Electron Microscopy

For electron microscopy (EM) of whole cells, the specimens were prepared by the pseudoreplica technique as described by Martin et al. (75), using 0.5% uranyl acetate for negative staining. For immunoelectron microscopy (IEM), N16 cells were incubated with a 1:100 dilution of rabbit antiserum overnight at 4°C, washed with pH 7.2 PBS, and incubated (2 h, 25°C) with a 1:10 dilution of gold-conjugated goat anti-rabbit IgG (Auroprobe EM GAR G10, Janssen Life Sciences Products, Piscataway, NJ). Then the gold-labeled cells were prepared for EM and negatively stained as described above.

For thin section IEM colloidal gold (diameter, approximately 15 nm) was prepared by citrate reduction of chlorauric acid (43) and coupled to goat anti-mouse IgG by the method of Horisberger and Rosset (54).

N16 cells were grown in brain heart infusion broth (48 h, 37°C), then harvested and washed with pH 7.2 PBS by vacuum filtration on Nucleopore filters (45 μ pore size). The cells were reacted with monoclonal antibody by floating the filters on hybridoma culture supernatant (immune sample) or on myeloma cell line P3X63Ag8 culture supernatant (negative control) for 1 h at 25°C. The filters were washed extensively with PBS, and then floated on gold-conjugated goat anti-mouse IgG for 1 h at 25°C. Filters were washed in PBS, fixed for 1 h in 2.5% glutaraldehyde in PBS with 0.5% tannic acid, post-fixed in 1% OsO_4, dehydrated, embedded in Spurr's resin, and sectioned. Thin sections were stained with uranyl acetate and lead citrate and examined on a JEOL 100-CX electron microscope.

Hemagglutination

The assay described below, as performed with unstabilized erythrocytes (RBC), is basically that of Costello et al. (29).

Preparation of neuraminidase-treated RBC. Human type O blood was drawn in anticoagulant and diluted with four volumes of pH 7.2 PBS. RBC were collected by centrifugation at 750 x g for 10 min and washed twice more in the same manner. A 15% (v/v) suspension of RBC in neuraminidase (30 µg per ml; Cat. No. N2876 type V neuraminidase from Clostridium perfringens, Sigma Chemical Co.) in pH 5.0 PBS was incubated in a 37°C waterbath for 2 h. Neuraminidase-treated RBC (NTRBC) were washed three times and suspended in TBS containing 0.4% BSA. NTRBC had to be used within a day or two, unless they were stabilized by treatment with formaldehyde or glutaraldehyde.

Formaldehyde stabilization of NTRBC. After neuraminidase treatment an aliquot of NTRBC was washed in pH 7.2 PBS to remove the enzyme and acid buffer. Then a 10% (v/v) suspension of NTRBC in PBS, pH 7.2, was mixed with an equal volume of 3.7% formaldehyde. The suspension was incubated at 25°C with occasional stirring for 4-6 h, then at 37°C with continuous stirring for 14-18 h. The formaldehyde-stabilized NTRBC were washed 4X with 10 volumes of TBS and 3X with TBS containing 0.4% BSA. They were stored at 4°C in TBS-BSA. These cells were evaluated versus freshly prepared and aged preparations of NTRBC that had not been treated with formaldehyde to determine whether formaldehyde stabilization prolonged the "life" of the NTRBC.

Glutaraldehyde stabilization of NTRBC. A 1-2% (v/v) suspension of RBC in cold 1% glutaraldehyde in pH 8.2 PBS was incubated in an ice

bath for 30 min with intermittent mixing (2). The glutaraldehyde-treated RBC (G-RBC) were collected by centrifugation (750 x g, 10 min) and washed once in saline. To block potentially reactive free aldehyde groups, a 10% (v/v) suspension of G-RBC in 0.1 M glycine was incubated at 25°C for 1 h. Then G-RBC were washed 5-10 times in TBS. They were stored in TBS with 0.4% BSA at 4°C. Glutaraldehyde stabilization could be used either before or after neuraminidase treatment.

Test procedure. Starting with a bacterial cell suspension equivalent to A_{650} = 2.0, serial two-fold dilutions in TBS with 0.4% BSA were made in a U-bottom microtiter plate, leaving 25 µl per well. Then 25 ul of a 1% (v/v) suspension of NTRBC in TBS-BSA were added to each well and mixed for 1 min. The reactions were read immediately and after overnight incubation at ambient temperature. To test for lactose-reversibility, 50 µl of 0.04 M lactose (0.02 M final concentration) were added. Alternatively, the effects of various inhibitors were tested by adding 50 µl of inhibitor to the bacterial cells prior to the addition of NTRBC.

Coaggregation

The protocol described below is based on the methods of McIntire et al. (82) and Cisar et al. (18).

Tube assay. Bacterial cell suspensions in TBS were adjusted to A_{650} = 2.0–2.1 (1-cm cuvette, Beckman Model 25 spectrophotometer). In a 10 x 75 mm test tube 0.2 ml of an actinomycete cell suspension was mixed with an equal volume of a streptococcal cell suspension. For controls, 0.2 ml of buffer was added in place of one of the cell suspensions. The suspensions were mixed by vortexing, then incubated

overnight at ambient temperature. They were mixed again before the reactions were graded on a scale of 0 to 4+ : 0 = no visible aggregates; 1+ = small uniform aggregates in suspension; 2+ = definite aggregates that did not settle immediately; 3+ = large aggregates that settled rapidly, leaving some turbidity in the supernatant; 4+ = large aggregates that settled immediately, leaving a clear supernatant.

Microtiter plate assay. A microtiter plate coaggregation assay was developed to replace the standard tube assay. The procedure was similar to that described for the tube assay, except that 25 µl of each cell suspension were mixed in a U-bottom microtiter plate.

Bacterial Agglutination

In a U-bottom microtiter plate serial dilutions of the antibody samples were made in TBS containing BSA (4 mg/ml). Normal sera and diluent were used as negative controls. To 25 µl of antibody were added 25 µl of a suspension of bacterial cells in TBS adjusted to A_{650} = 1.0 (1 cm cuvette, Beckman Model 25 spectrophotometer), and the plate was shaken for 1-2 min. Reactions were read after overnight incubation at 25°C (14).

Radioimmunoassay

A solid-phase radioimmunoassay (RIA) was developed as a rapid, sensitive screening assay for detection of monoclonal antibodies to surface antigens of N16 and other isolates. Polyvinyl chloride microtiter plates (U-bottom, Dynatech Laboratories, Inc.) were coated with formalin-killed whole cells by placing 25 µl of a No. 4 McFarland cell suspension in each well for 1 h at 25°C. Excess antigen was rinsed out, and free sites were blocked by the addition of a few drops of 10% agamma horse serum to each well. The antigen-

coated wells were incubated (1 h, 25°C) with 25 µl of undiluted hybridoma culture supernatant. The wells were washed three times with PBS containing 1% fetal calf serum and incubated (1 h, 25°C) with 25 µl of ^{125}I-labeled rabbit anti-mouse IgG (heavy and light chain specific) containing 30,000-50,000 counts per minute (cpm). After the unbound radiolabeled second antibody had been washed away, individual wells were cut out, and the bound radioactivity was measured in a gamma counter. Reactions in which ^{125}I-cpm were at least twice that of the negative control were considered to be positive. Culture supernatants from the myeloma lines used for fusion served as negative controls. Murine antisera were used as positive controls if positive culture supernatants or ascites fluids were not available.

Indirect Enzyme Immunoassay

Indirect enzyme immunoassays (EIA) were performed in Immulon 2 96-well flat-bottomed polystyrene microtiter plates (Dynatech Laboratories, Inc.). Wells were coated with antigens by placing 50 µl of whole cells or soluble antigens optimally diluted in TBS in each well and drying at 37°C. Antigen-coated plates were stored at ambient temperature until used. Plates were washed once with blocking buffer (PBS with 1% BSA), then 3 times with PBS with 0.05% polyoxyethylene sorbitan monolaurate (Tween 20) (PBST). Antibodies diluted in PBST were added to the wells (25 µl per well) and incubated at 25°C for 1 h. Plates were washed 3 times with PBST. Then 100 µl of horseradish peroxidase-labeled goat anti-rabbit or mouse IgG optimally diluted in PBST were added and incubated at 25°C for 1 h. After the excess conjugate had been removed by washing, 200 µl of substrate solution (0.1 mg of ortho-phenylenediamine and

2 µl of 3% H_2O_2 per ml of citrate-phosphate buffer, pH 5.0) were added and incubated at 25°C for 15-30 min. Reactions were stopped with 25 µl/well of 4 M H_2SO_4, and the absorbance at 490 nm was read on a Dynatech MR 600 Microplate Reader (Dynatech Laboratories, Inc.). Absorbance readings >0.2 above the negative control were considered positive.

Enzyme Immunodot Assay

Antigens were affixed to strips of nitrocellulose by applying 2-µl drops at 5-mm intervals and allowing them to dry at 25°C (79). The antigen-coated strips were processed by enzyme immunoassay essentially as described by Tsang et al. (99). The strips were washed 4 times for 5 min each time with PBS with 0.3% Tween 20 (PBSTW) with a quick rinse in deionized water after each PBSTW wash. The antigen-coated strips were immersed in antibodies diluted in PBSTW and incubated with gentle agitation for 1 h at 25°C. Then the strips were washed as before to remove unbound antibodies. The strips were covered with the appropriate peroxidase-conjugated anti-Ig and incubated for 1 h at 25°C. Unbound conjugate was removed by washing as described above, followed by a final wash with PBS. The strips were incubated with substrate (50 mg of 3,3'-diaminobenzidine and 10 µl of 30% H_2O_2 per 100 ml of PBS) for 10 min or until the spots had developed the desired degree of intensity. The reaction was stopped by rinsing the strips thoroughly with water.

Immunodiffusion

Immunodiffusion was performed in 1% agarose in PBS with wells formed by use of a microimmunodiffusion template (89).

Laurell Rocket Immunoelectrophoresis

Laurell rocket immunoelectrophoresis (LRI) was performed as described by Powell et al. (90) in 0.75% agarose in 0.043 M sodium barbital buffer, pH 8.3, at a constant current of 8 mA per 50 mm x 75 mm slide. Gels were dried and stained with 0.5% Coomassie brilliant blue R-250 in ethanol:glacial acetic acid:water (4.5:1.0:4.5).

Crossed Immunoelectrophoresis with Autoradiography

Glass slides (50 mm x 75 mm) were coated with 7 ml of 0.75% agarose in barbital buffer (0.0375 M barbital, 2 mM calcium lactate, 0.05% NaN_3, pH 8.6). After the antigen wells were cut and filled, the gels were electrophoresed at 8 mA constant current for 1-3 h for separation of antigens in the first dimension. Then the gel above the antigen wells was replaced with 3 ml of agarose containing an unlabeled rabbit antiserum raised against whole cells and an ^{125}I-labeled Ig (50). After electrophoresis in the second dimension overnight at 4 mA constant current, gels were washed, dried, and exposed to Kodak XAR-2 film with a Dupont Cronex Lightning Plus intensifying screen at -70°C. Gels were stained with Coomassie brilliant blue R-250 and photographed.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Socium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Tsang et al. (102). Samples were prepared to obtain final concentrations of 1% SDS, 0.9 M urea, 0.05% bromophenol blue, 8% glycerol, and 0.25 mg total protein per ml. Some samples also received dithiothreitol (DTT) or 2-mercaptoethanol for reduction of disulfide bonds. Samples were heated at 37°C for 30 min, at 65°C for 15 min, or at 100°C for

5 min. Gels consisting of a 5-20% linear gradient resolving gel and a 3% stacking gel 0.75 mm thick were cast using the Pharmacia GSC-2 177 mm x 200 mm vertical slab system (Pharmacia). The gels were electrophoresed in the Pharmacia GE 2/4 LS electrophoresis chamber using a discontinuous buffer system with 0.424 M Tris-HCl, pH 9.18, in the lower reservoir and 0.04 M boric acid, 0.04 M Tris, 0.1% SDS in the upper reservoir. Generally 0.25 µg of protein per mm width of sample lane was applied. Either high molecular weight markers (Bio-Rad) and low molecular weight markers (Pharmacia) or prestained protein molecular weight standards (Bethesda Research Laboratories, Gaithersburg, MD) were included in each gel (101). After electrophoresis protein bands were visualized by the silver stain as described by Tsang et al. (100), which is a combination of the methods of Merril et al. (85) and Morrissey (88).

Immunoblot

SDS-PAGE resolved proteins were transferred electrophoretically to nitrocellulose (0.2 µ, Schleicher and Schuell, Keene, NH); then antigens were visualized by an indirect enzyme immunoassay, using peroxidase conjugates (Bio-Rad Western blotting grade) and diaminobenzidine/ H_2O_2 as the substrate. These methods have been described in detail by Tsang et al. (99,102). For detecting unstained molecular weight markers or other proteins on blots, nitrocellulose strips were stained with India ink (52) or Aurodye forte (Janssen Life Sciences Products, Piscataway, NJ).

RESULTS AND DISCUSSION

Monoclonal Antibodies

Three different hybridomas secreting antibodies to A. naeslundii serotype 3 strain N16 were produced. Hybridomas 3B5.A1 and 2A3.B3, both obtained from the fusion with the non-Ig-producing Kearney myeloma cell line, secreted IgG_{2a} and IgG_3 monoclonal antibodies, respectively. The third hybridoma, 2B5.B6, which was a product of the fusion with an IgG_1(k)-secreting myeloma cell line, secreted IgM monoclonal antibodies along with the Ig chains of the myeloma parent.

Preliminary indications of differences in specificity and the range of reactivity of each of the three monoclonal antibodies were obtained by radioimmunoassay, using whole cells as antigens. When tested against a panel of ten isolates of A. naeslundii serotype 3, monoclonal antibodies 3B5.A1 and 2A3.B3 reacted with all 10 isolates, whereas monoclonal antibody 2B5.B6 reacted with only 4 of the 10 isolates (Table 1). On the basis of these results, monoclonal antibodies 3B5.A1 and 2A3.B3 appeared to have potential as serotyping reagents, provided that they did not also react with heterologous serotypes; monoclonal antibody 2B5.B6 had too narrow a range of reactivity to be useful for serotyping, unless it could be used as an epidemiological tool for fine typing below the serotype level. To determine whether or not the monoclonal antibodies would cross-react with other organisms, the panel of cells for the RIA was expanded to include 51 heterologous isolates: 17 isolates of A. naeslundii that

TABLE 1. Summary of RIA results for isolates within the N16 serogroup

Isolate	Monoclonal antibody		
	3B5.A1	2A3.B3	2B5.B6
N16	+[a]	+	+
WVU1267	+	+	-
WVU1468	+	+	-
WVU1527	+	+	+
WVU1528	+	+	+
W1629	+	+	-
W2273	+	+	-
W2821	+	+	+
UF92	+	+	-
UF524	+	+	-

[a] + = ^{125}I-cpm > ^{125}I-cpm of negative control x 2; - = ^{125}I-cpm < ^{125}I-cpm of negative control x 2 for the average ^{125}I-cpm of samples run in duplicate. ^{125}I-cpm of negative controls ranged from 53 to 78, whereas ^{125}I-cpm of + reactions ranged from 1919 to 6283.

did not belong to the N16 serogroup (serotype 3), 13 isolates of A. viscosus, 8 isolates of A. israelii, and 13 isolates of less closely related species. When assayed using polyclonal antisera, A. naeslundii and A. viscosus are closely related antigenically, and antisera raised to whole cells of N16 could be expected to cross-react with A. viscosus serotype 2 in particular. However, monoclonal antibody 3B5.A1 did not react with any heterologous species or serotypes (Table 2). On the other hand, monoclonal antibody 2A3.B3 cross-reacted with 7 of 17 non-serotype 3 A. naeslundii isolates, as well as 7 of 11 A. viscosus serotype 2 isolates. However, the ^{125}I-cpm for all but one of these cross-reactions were less than 34% of the ^{125}I-cpm of the least reactive A. naeslundii serotype 3 isolate. Monoclonal antibody 2B5.B6 cross-reacted with 4 of 17 isolates representing other serotypes of A. naeslundii, and these cross-reactions were as strong as the homologous reactions. None of the three monoclonal antibodies reacted with isolates of A. israelii, A. odontolyticus, A. pyogenes, A. propionica, R. dentocariosa, P. avidum, or Bacteroides gingivalis. These results suggested that monoclonal antibody 3B5.A1 recognizes a serotype-specific antigenic determinant but that monoclonal antibodies 2A3.B3 and 2B5.B6 see epitopes common to other species or serotypes.

The location of the epitopes to which the monoclonal antibodies were binding was presumed to be the cell surface because whole cells were used as immunogens and as antigens in immunoassays to measure monoclonal antibody activity. The most immunogenic and biologically significant surface components of A. naeslundii and A. viscosus isolates are their fimbriae. Electron microscopy of A. naeslundii N16

TABLE 2. Summary of the specificities of three monoclonal antibodies to N16 as determined by RIA

Species/serotype	No. isolates tested	Monoclonal antibody 3B5.A1	2A3.B3	2B5.B6
Actinomyces naeslundii/3	10	10[a]	10	4
A. naeslundii/1, 2, 4	17	0	7	4
A. viscosus/1	2	0	0	0
A. viscosus/2	11	0	7	0
A. israelii/1	6	0	0	0
A. israelii/2	2	0	0	0
A. odontolyticus	3	0	0	0
A. pyogenes	1	0	0	0
Arachnia propionica	3	0	0	0
Rothia dentocariosa	2	0	0	0
Propionibacterium avidum	3	0	0	0
Bacteroides gingivalis	1	0	0	0

[a] Number of isolates that gave positive reactions, as defined in Table 1. The range of ^{125}I-cpm for positive reactions was as follows: 2547-6283 for 3B5.A1 against homologous isolates; 1919-6025 for 2A3.B3 against homologous isolates; 2097-2628 for 2B5.B6 against homologous and heterologous isolates. Except for 3207 ^{125}I-cpm against A. naeslundii W1250, 2A3.B3 gave 161-649 ^{125}I-cpm against heterologous serotypes of A. naeslundii and 180-328 ^{125}I-cpm against A. viscosus serotype 2 isolates.

cells revealed that they possess abundant fimbriae (Figure 1). The location and density of the epitopes recognized by each monoclonal antibody, as determined by EM of N16 cells immunolabeled with colloidal gold, are shown in Figure 2. All three monoclonal antibodies bound to epitopes residing on the fimbriae, but based on the far greater number of gold particles seen on cells immunolabeled with MAb 3B5.A1, the 3B5.A1 epitope appeared to be more abundant than those recognized by MAbs 2A3.B3 and 2B5.B6.

All three of the MAbs against N16 fimbriae were used on occasion in later experiments. However, most of this study focused on the use of MAb 3B5.A1 because preliminary evaluations indicated that (a) it reacted with all 10 A. naeslundii serotype 3 strains by RIA, (b) it appeared to recognize a unique, serotype-specific epitope, and (c) on the basis of the density of immunogold labeling, it appeared to recognize an epitope that was more abundant than those of the other two MAbs.

Evidence for the Presence of Both Types of Fimbriae on A. naeslundii Serotype 3 Strains

Evidence for the Presence of Type 1 Fimbriae

Although this study did not include any functional assays to demonstrate the presence of type 1 fimbriae on N16 or other A. naeslundii serotype 3 isolates, Clark et al. (23,25,26,28) have presented evidence that 3 of the 10 strains listed in Table 1 possess type 1 fimbriae. In one study they showed by EM that N16 and UF92 cells had fimbriae; both strains adsorbed very well to saliva-coated hydroxyapatite (SHA) relative to the adsorption of T14V, 100% for N16 and 84% for UF92; and their adherence to SHA could be blocked by purified T14V fimbriae (26). In another study N16 and G1468 (WVU1468)

Figure 1. Electron micrograph of A. naeslundii N16 cells negatively stained with 0.5% uranyl acetate. Note abundant fimbriae on the cell surface. Bar, 1 μm.

Figure 2. Electron micrographs of thin sections of immunogold labeled A. naeslundii N16 cells demonstrating the binding of the monoclonal antibodies to fimbrial epitopes. The cells were incubated with MAb-containing culture supernatants of myeloma or hybridoma cell lines and then with goat anti-mouse IgG conjugated to colloidal gold (diameter, 15 nm). Bar, 0.25 μm.
A) P3X63Ag8, negative control; B) MAb 2B5.B6; arrows indicate gold particles; C) MAb 2A3.B3; D) MAb 3B5.A1; E) MAb 3B5.A1; F) MAb 3B5.A1

A
B

Figure 2. --continued

C
D

Figure 2. --continued

E

F

adsorbed to SHA better than T14V, despite the fact that each had a lower hydrophobic index than T14V (25). N16 and WVU1468 reacted in an immunodot EIA with rabbit IgG specific for the T14V type 1 fimbriae (28), and rabbit IgG anti-T14V type 1 fimbriae was able to partially inhibit adsorption of N16 cells to SHA and give 91% inhibition for WVU1468 (23,28). The ability of N16, WVU1468, and UF92 to adsorb well to SHA and the fact that T14V fimbriae or antibodies to T14V type 1 fimbriae can inhibit the adsorption suggests that these A. naeslundii serotype 3 strains possess type 1 fimbriae. Immunological assays performed in this study (data to be presented later) showed that all of the A. naeslundii serotype 3 strains listed in Table 1 have type 1 fimbriae.

Evidence for the Presence of Type 2 Fimbriae

Hemagglutination. Many human strains of A. viscosus and A. naeslundii can agglutinate human types A, B, O, and AB erythrocytes (29,37). With untreated RBC, hemagglutination occurs immediately at $4^{o}C$, but the reaction takes longer at room temperature or $37^{o}C$. With neuraminidase-treated RBC, hemagglutination occurs immediately at all three temperatures (29). Hemagglutination by A. viscosus and A. naeslundii cells can be completely reversed by lactose. The mechanism proposed for this reaction involves two steps: 1) the unmasking of ß-galactoside-containing receptors on the RBC when terminal sialic acid residues are removed by neuraminidase released from Actinomyces cells, and 2) the multivalent binding of these receptors by multiple low affinity, lactose-reversible lectin sites on the surface of the actinomycete (29,37). Since the lactose-reversible lectin activity is a function of type 2 fimbriae (20), HA can be used

to determine whether or not Actinomyces cells have type 2 fimbriae.

Costello et al. (29) reported that N16 and WVU820 (N16) produced neuraminidase and agglutinated human RBCs as described above. On the other hand, Ellen et al. (37) reported that strain 820, which is presumably WVU820 (N16), did not agglutinate non-sialidase-treated horse, sheep, guinea pig, or human RBCs. Since Ellen et al. measured HA after only a 5 min incubation, the inability of WVU820 to agglutinate RBCs was probably a result of not allowing sufficient time for the WVU820 neuraminidase to remove the terminal sugar blocking the receptor.

In this study the 10 A. naeslundii serotype 3 strains listed in Table 1 were tested for HA activity. Preliminary experiments were performed with unfixed NTRBC, but unfixed NTRBC were found to be so unstable that they generally had to be used within 24 h of preparation. Even then they often gave weak HA reactions or lysed during assays. In an effort to improve their shelf-life, NTRBC were fixed with formaldehyde or glutaraldehyde and compared to unfixed NTRBC. HA reactions with fixed NTRBC were stronger than with unfixed, and glutaraldehyde-fixed RBC were superior to formaldehyde-fixed cells. Not only did glutaraldehyde-fixed NTRBC agglutinate better than unfixed cells, but they also had a shelf-life at 4°C that could be measured in years instead of hours. The characteristics of the agglutination reactions between N16 cells and human RBC (in terms of the effects of temperature, incubation time, etc. as reported by Costello et al.) were the same for glutaraldehyde-fixed RBC or NTRBC as for unfixed RBC or NTRBC, except that HA with fixed cells was stronger and, therefore, more difficult to reverse. All 10

A. naeslundii serotype 3 isolates agglutinated G-NTRBC, and all of the HA reactions were reversed by lactose. Twelve sugars and EDTA were tested for their ability to inhibit HA by N16 cells. Glucose, fructose, mannose, sucrose, xylose and α-methyl galactoside exhibited no inhibition of HA at concentrations of inhibitor ≤ 25 mM. Of the substances that did inhibit at concentrations < 25 mM, the relative potency of the inhibitors was as follows: EDTA > lactose > ß-methyl galactoside > talose > fucose > galactose > N-acetyl galactosamine. These results on the relative potency of inhibitors of the N16 HA reaction are similar to the results of Ellen et al. (37) for the inhibition of HA by A. naeslundii serotype 2 W752 cells and also the results of McIntire et al. (81,82) for inhibition of coaggregation between T14V and Streptococcus sanguis strain 34 cells. N16 cells also agglutinated sheep and guinea pig RBC, and HA was completely inhibited with EDTA or lactose at a final concentration of 25 mM.

Coaggregation. Many human strains of A. viscosus and A. naeslundii can agglutinate certain strains of oral streptococci (18,19,36,69,81,82). These coaggregation reactions involve different mechanisms for interaction, one of which is the binding of a carbohydrate moiety on S. sanguis 34 cells by a lectin on Actinomyces cells in a reaction that can be completely reversed by lactose (18,69,81,82). Coaggregation, like hemagglutination, is a function of type 2 fimbriae (20,92), so it also can be used to determine whether or not Actinomyces cells have type 2 fimbriae.

Cisar et al. (18) showed that WVU820 (N16) and W1527 (WVU1527) exhibited lactose-reversible coaggregation with S. sanguis 34 cells.

Their observations were confirmed in this study, using both the tube and microtiter plate coaggregation assays (data not shown).

In summary, there was ample evidence from our preliminary studies to suggest that N16 and other A. naeslundii serotype 3 strains had both type 1 and type 2 fimbriae. The next step was to find a good way to remove the fimbriae from the cells and obtain crude soluble fimbrial extracts suitable for purification of fimbriae and for use as antigens in immunological assays.

Antigen Preparation: Results of Preliminary Experiments

In the early stages of this research several different methods for preparing soluble fimbrial extracts of N16 cells were tried. First, the method of Wheeler and Clark (106) for purifying VA-1 fibrils (type 1 fimbriae) from A. viscosus T14V cells by ammonium sulfate fractionation of French pressure cell supernatants was attempted. Laurell rocket immunoelectrophoresis of the N16 fractions prepared in this manner, i.e. 10, 20, 30, 40, and 50% (w/v) ammonium sulfate saturated samples, revealed multiple antigens in each fraction when antiserum to N16 whole cells was employed. When these fractions were used to coat wells in the RIA, MAb 3B5.A1 gave ^{125}I-cpm with the 20% and 30% ammonium sulfate fractions that were 5-8 times higher than those obtained with the 10%, 40%, or 50% fractions; thus the 20% and 30% fractions appeared to be enriched for the antigen that 3B5.A1 recognizes. Each fraction was examined by transmission electron microscopy for the presence of fimbriae, but the 20% fraction was the only one in which fimbriae were readily observed.

Although the French pressure cell was an effective means for removing fimbriae from cells with minimal cell lysis, access to this

equipment was not readily available. So, several other methods for extracting fimbrial antigens were investigated: Lancefield extraction, sonication, Virtis homogenization, and extraction with 8 M lithium chloride. N16 cells, before and after being treated by these various procedures, were tested for lectin activity by hemagglutination. Since type 2 fimbriae of Actinomyces exhibit lectin activity, the efficacy of each treatment of the cells in removing type 2 fimbriae could be compared in this manner; presumably, type 1 fimbriae would be removed also. Sonication and Lancefield extraction reduced the lectin activity of N16 cells from 2+ to 0, whereas Virtis homogenization and extraction with 8 M lithium chloride had little, if any, effect on the lectin activity. Electron microscopy confirmed that sonication and Lancefield extraction were much more effective than Virtis homogenization or lithium chloride in removing fimbriae from N16 cells. The observation that treatment of N16 cells with 8 M LiCl had no detectable effect on the fimbriae is contrary to the observation of McIntire et al. (82) that A. viscosus T14V cells treated in such a manner lost their fimbriae and, consequently, their ability to coaggregate with S. sanguis 34.

The total protein and total carbohydrate extracted per gram wet weight of cells by these procedures are compared in Table 3. Sonication released greater than 15 times more protein than either Virtis homogenization or lithium chloride extraction, and the protein to carbohydrate ratio was greater than 1.0 in each case. For Lancefield extraction the protein to carbohydrate ratio was only 0.14. Lancefield extraction was eliminated from consideration as a method for extracting fimbriae for subsequent purification because it

released 7 times more carbohydrate than protein and because of the likelihood of protein denaturation resulting from acid hydrolysis. However, it was the preferred method for preparing soluble antigens for immunoelectrophoresis because (a) it was the most convenient way to extract antigens from a large number of strains, and (b) it invariably gave strong, symmetrical type 1 and type 2 fimbrial rockets in LRI and XIEP.

TABLE 3. Total protein and total carbohydrate released from N16 cells by several methods for extracting fimbriae

Method of extraction	CHO[a]	Protein[b]
Continuous flow sonication	5.0	11.2
Virtis homogenization	0.5	0.6
8 M LiCl	0.3	0.4
Lancefield extraction[c]	22.8	3.1

[a] mg total carbohydrate released per g wet weight of cells.

[b] mg total protein released per g wet weight of cells.

[c] A different method was used to determine the wet weight of cells subjected to Lancefield extraction, so the quantity of CHO and protein extracted by that method cannot be compared directly to the quantities extracted by the other methods.

Although French pressure cell shearing and continuous flow sonication were my first and second choices, respectively, for obtaining crude extracts for purification of N16 fimbriae, lack of access to the appropriate equipment at the proper time ultimately dictated that the initial crude fimbrial extracts be obtained by a batch method of sonication. Later in the study, a French pressure cell extract was also processed.

Identification of N16 Type 1 and Type 2 Fimbriae in Crude Antigen Extracts by XIEP-A

Immunoelectron microscopy had been used to demonstrate that the 3B5.A1 epitope was located on N16 fimbriae, but whether those fimbriae were type 1 or type 2 fimbriae had not yet been established. Analysis of N16 crude sonicates by XIEP against rabbit antiserum to whole cells revealed the presence of two major antigens that were thought to correspond to type 1 and type 2 fimbriae (Figure 3, upper panels). Cisar et al. (14) had shown that in XIEP Ag 1 (type 1 fimbriae) of T14V migrated closer to the anode than Ag 2 (type 2 fimbriae). Whether the type 1 and type 2 fimbriae of all Actinomyces strains maintained the same relative electrophoretic mobilities was not known. In fact, since crude sonicates of N16 cells contained other antigens besides fimbrial antigens, and the antiserum used to precipitate the antigens was not specific for fimbriae, the two N16 rockets did not necessarily have to be fimbrial antigens.

In order to establish the identity of the two N16 rockets and to determine which type of fimbriae MAb 3B5.A1 recognized, fimbriae-specific ^{125}I-Ab was incorporated into the Ab-containing portion of the gel along with the non-radiolabeled polyspecific antiserum. When ^{125}I-Ab specific for T14V type 1 fimbriae was used, autoradiography of the gel demonstrated that the N16 rocket closer to the anode corresponded to type 1 fimbriae (Figure 3A). When the gel contained ^{125}I-MAb 3B5.A1, autoradiography revealed that MAb 3B5.A1 bound to the other rocket (Figure 3B). Since (a) MAb 3B5.A1 binds to fimbriae, (b) it did not bind to type 1 fimbriae, and (c) only two types of Actinomyces fimbriae have been identified, by a process of elimination it was determined that the antigen that migrated more

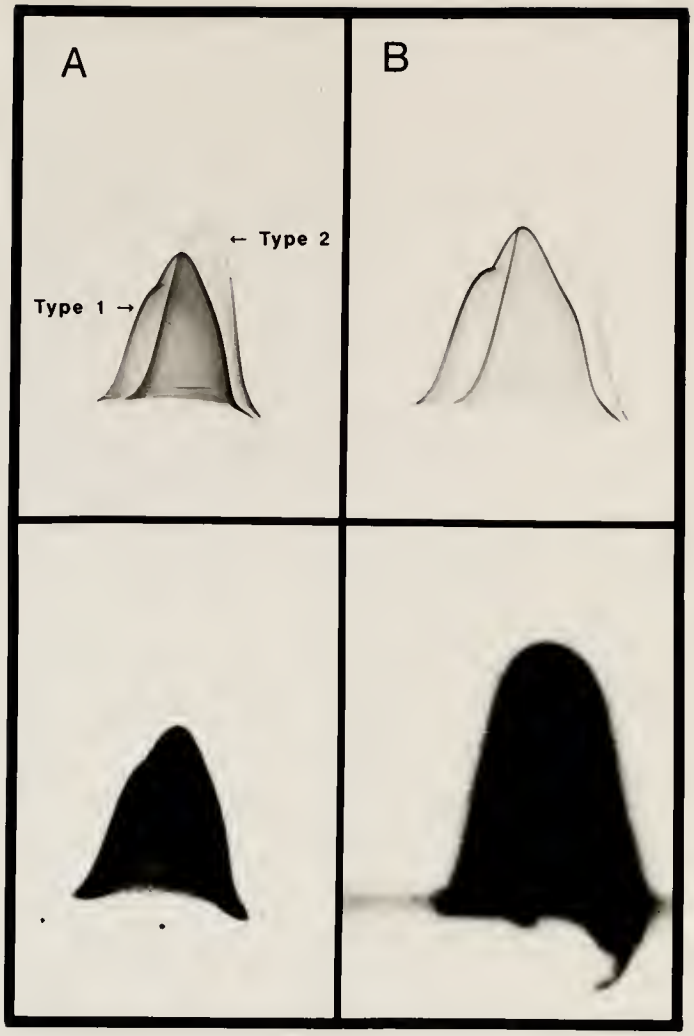


Figure 3. Identification of A. naeslundii N16 type 1 and type 2 fimbriae by XIEP-A. N16 cell surface antigens extracted by sonication were separated by electrophoresis in the first dimension with the anode to the left. Then they were precipitated by electrophoresis (at a right angle to the first dimension) into gel containing R29 rabbit antiserum against N16 whole cells and ^{125}I-labeled IgG specific for fimbriae. Two rockets precipitated by R29 are seen in the Coomassie-stained gels (upper panel); the corresponding autoradiographs (lower panel) indicate that the rocket closer to the anode represents type 1 fimbriae, whereas the other rocket represents type 2 fimbriae. In this and all subsequent figures of XIEP patterns, the anode is on the left for electrophoresis in the first dimension and at the top for electrophoresis in the second dimension. The type 1 fimbrial rocket is always on the left and the type 2 fimbrial rocket on the right.
A) ^{125}I-R59 specific for the type 1 fimbriae of A. viscosus T14V binding to the type 1 fimbriae of N16;
B) ^{125}I-MAb 3B5.A1 binding to the type 2 fimbriae of N16.

slowly toward the anode was type 2 fimbriae and that MAb 3B5.A1 was specific for type 2 fimbriae.

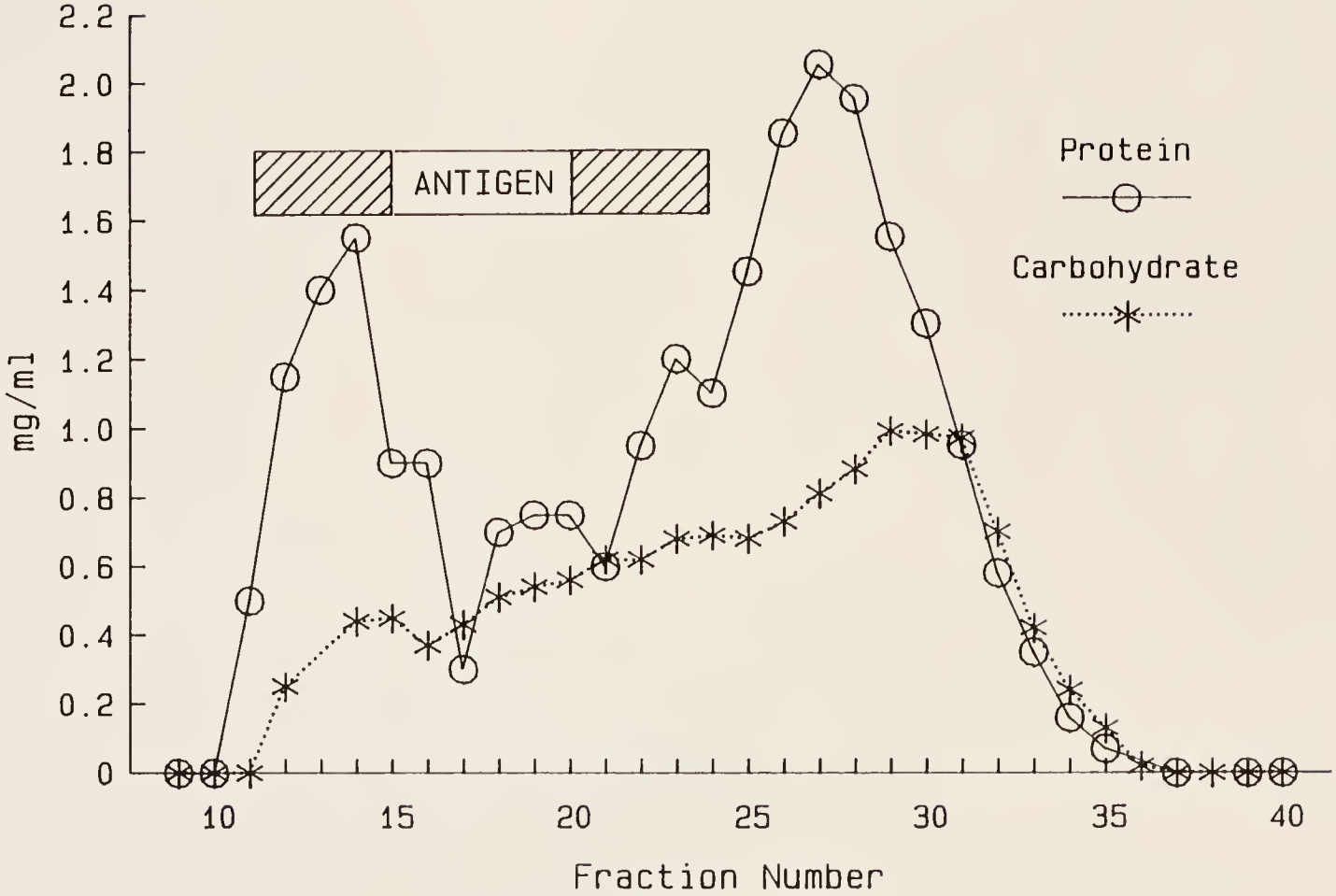
Immunological detection of type 2 fimbriae throughout various purification procedures and purification of type 2 fimbriae by immunoaffinity chromatography were made possible by the availability of MAb 3B5.A1. Also, XIEP-A proved to be a useful technique for monitoring antigen fractions for the presence of both types of fimbriae, as well as non-fimbrial antigens.

Purification of N16 Type 2 Fimbriae From a Crude Sonicate Batch A

Aliquots of the untreated control cells and the sonicated cells collected at different time intervals were assayed for lectin activity by HA to monitor the removal of type 2 fimbriae as measured by the decline in HA activity. Cells sonicated for only 30 sec had the same HA titer as the untreated control cells. With cells sonicated from 1 to 6 min total, the HA titers of the cells declined as the total sonication time increased. Most of the decrease in lectin activity had occurred after 2 min of sonication, but even after sonication for a total of 6 min, the cells still retained weak HA-positive activity. Electron microscopy on the cells sonicated for 6 min revealed that nearly all the cells had lysed and the cell walls appeared to be devoid of fimbriae.

When the crude 6 min sonicate was applied to the Bio-Gel A-5m column and eluted with TBS, two major protein-rich peaks were eluted, one just after the void volume and the other near the total volume (Figure 4). When the column fractions were dotted on nitrocellulose and reacted with MAb 3B5.A1 in an indirect EIA, fractions 11-24 gave

Figure 4. Elution profile for chromatography of A. naeslundii N16 crude sonicate on Bio-Gel A-5m. The hatched bar represents fractions strongly reactive with MAb 3B5.A1 as determined by immunodot EIA.


2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
mg/ml
ANTIGEN
Protein
Carbohydrate
10
15
20
25
30
35
40
Fraction Number

3-4+ reactions, but the intensity of the immunodots decreased sharply beyond fraction 24. Although fractions 18-24 also contained type 2 fimbriae, only fractions 11-17 (designated A-5m peak 1) were pooled, because fractions beyond the first major protein peak contained increasing levels of carbohydrate. The relatively high carbohydrate content of all the fractions in this batch probably was a reflection of the substantial amount of cell breakage that occurred when the cells were sonicated. When A-5m peak 1 was examined by XIEP against rabbit antiserum to N16 whole cells, both types of fimbriae and some nonfimbrial antigens were detected. A-5m peak 1 from Batch A was not purified further because another batch, less contaminated with cytoplasmic components, was prepared.

Batch B

Two minutes of sonication caused a 16-fold decrease in the 2+ HA endpoint relative to the untreated cell suspension, suggesting significant removal of type 2 fimbriae from the cells. Electron microscopy of negatively stained cells confirmed that sonication removed some but not all fimbriae from the cells. When the crude sonicate was precipitated with $(NH_4)_2SO_4$ at 50% saturation and again at 35% saturation, the supernatant fractions after dialysis and concentration were examined for the presence of fimbriae by XIEP. The results indicated that some of both types of fimbriae were lost to the supernatant fraction with each precipitation; however, no attempt was made to quantitate the amounts of fimbriae or total protein not precipitated.

The precipitate from 35% $(NH_4)_2SO_4$ saturation of the crude 2 min sonicate contained 390 mg total protein. This sample was

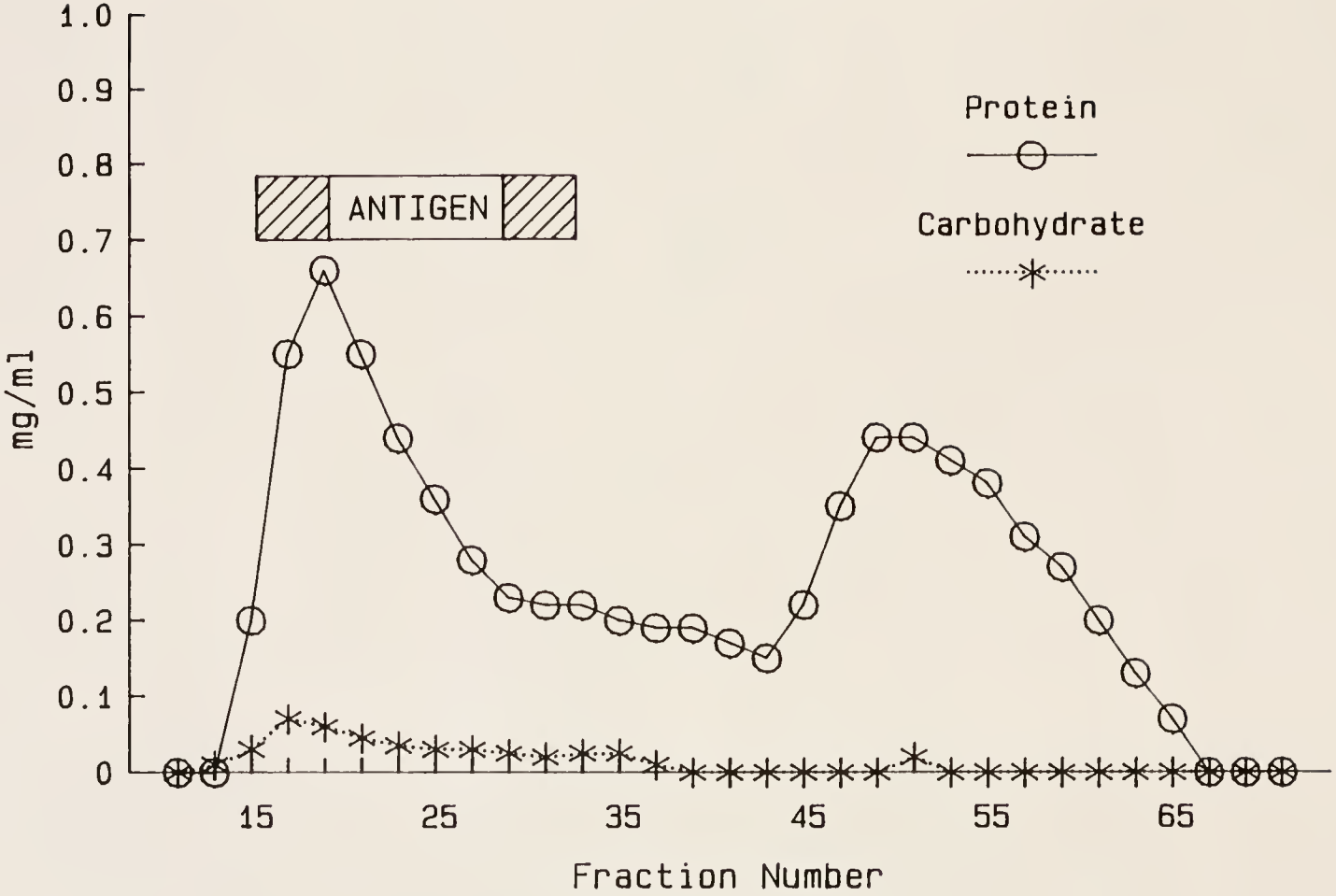
chromatographed in 5 separate aliquots on a Bio-Gel A-5m column; a representative elution profile is shown in Figure 5. This elution profile is similar to that obtained with Batch A (Figure 4), except that the Batch B fractions contained much lower levels of total carbohydrate. The first of two major protein-rich peaks that were eluted from the column contained most of the MAb 3B5.A1 reactive (type 2) fimbriae as determined by immunodot EIA on the column fractions. All of the fractions representing the first peak (peak 1) from each run, i.e., fractions 14-32 in Figure 5, were pooled; the A-5m peak 1 pool contained 159 mg total protein.

A 5 mg aliquot of the peak 1 pool was chromatographed on the MAb 3B5.A1 immunoaffinity column. Analysis of the column fractions by XIEP-A indicated that the unbound fraction resembled the starting material, which suggested overloading of the column, whereas the bound fraction appeared to contain only type 2 fimbriae.

The unbound fraction from the first immunoaffinity run was rechromatographed on the MAb column. This resulted in a reduction in type 2 fimbriae relative to type 1 in the unbound fraction and recovery of type 2 fimbriae in the bound fraction with only minor contamination with type 1 fimbriae (Figure 6). Thus, MAb 3B5.A1, which is specific for the type 2 fimbriae of N16, is an effective tool for the immunochromatographic purification of this structural entity.

The fimbrial immunoprecipitin arcs that were used to immunize rabbits for the production of polyclonal antibodies specific for N16 type 1 or type 2 fimbriae were obtained from these fractions, as shown in Figure 7 (refer also to Materials and Methods).

Figure 5. A representative elution profile for Bio-Gel A-5m chromatography of aliquots of the fraction obtained by precipitating A. naeslundii N16 crude sonicate with $(NH_4)_2SO_4$ at 35% saturation. The hatched bar represents fractions strongly reactive with MAb 3B5.A1 as determined by immunodot EIA.


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
mg/ml
ANTIGEN
Protein
Carbohydrate
15
25
35
45
55
65
Fraction Number

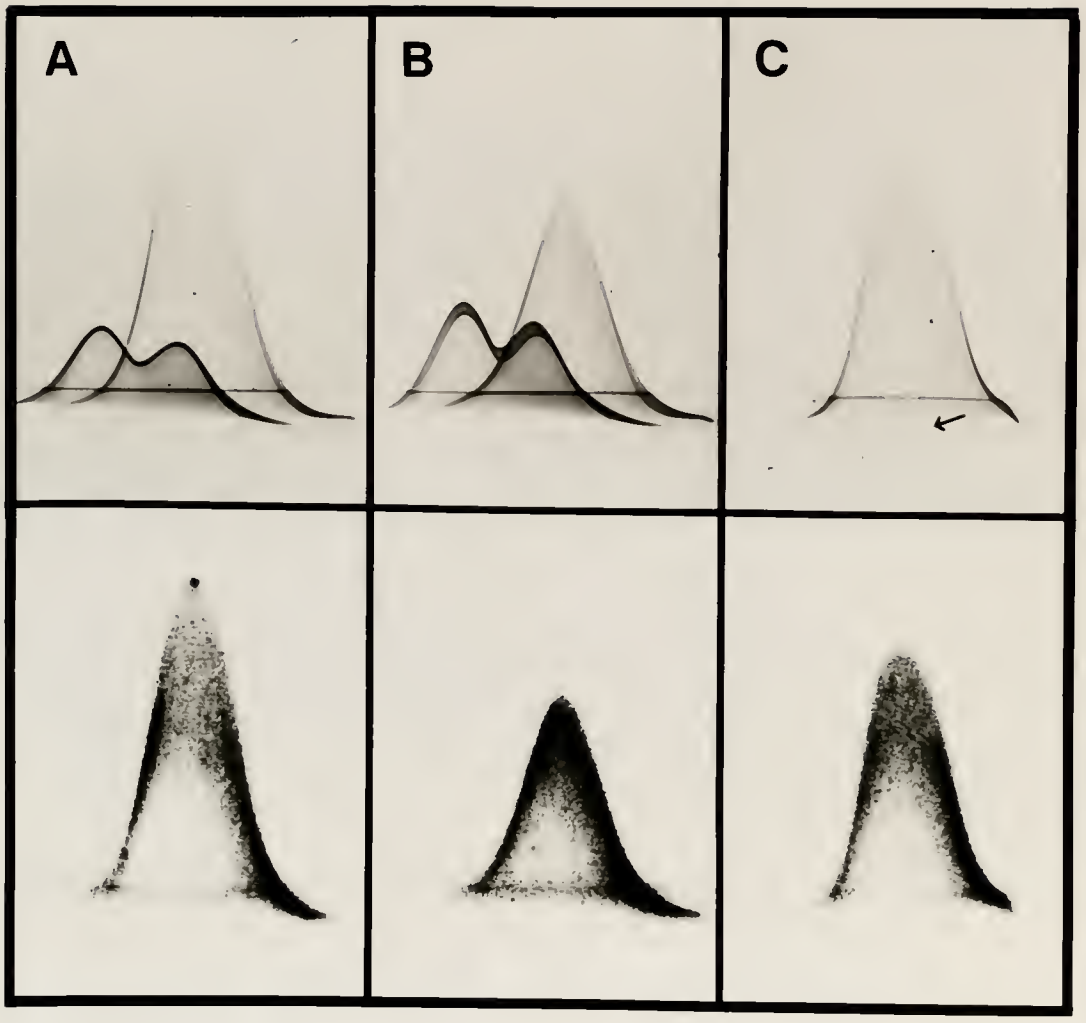


Figure 6. Results of immunoaffinity chromatography of A-5m peak 1 on the MAb 3B5.A1-Affi-Gel 10 column as demonstrated by XIEP-A. The antibody-containing portion of each gel included R29 antiserum against N16 whole cells and ^{125}I-labeled MAb 3B5.A1 specific for type 2 fimbriae. The Coomassie-stained gels (upper panel) show all the antigens precipitated by the polyspecific rabbit antiserum, whereas their corresponding autoradiographs (lower panel) identify the type 2 fimbriae.
A) A-5m peak 1, the starting material for immunoaffinity chromatography, contained both types of fimbriae; B) The unbound fraction also contained both types of fimbriae, but there was a reduction in type 2 fimbriae relative to type 1; C) The bound fraction contained mostly type 2 fimbriae with a barely detectable quantity of type 1 fimbriae (arrow).

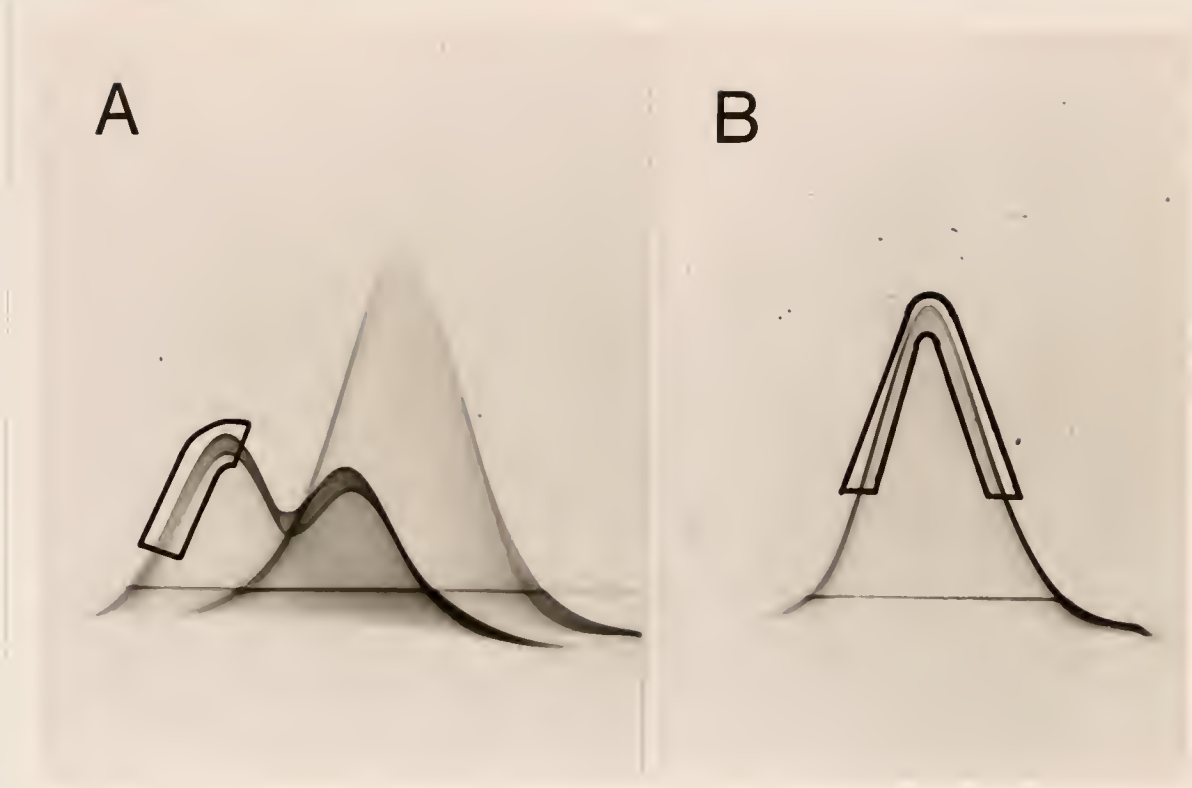


Figure 7. Source of immunogens for production of rabbit antisera monospecific for N16 type 1 or type 2 fimbriae. Segments of fimbrial immunoprecipitates were excised from the areas indicated on the XIEP gels. R29 against N16 cells was the precipitating antiserum in both gels.
A) Type 1 fimbrial immunoarcs for production of R10 antiserum;
B) Type 2 fimbrial immunoarcs for production of R2P antiserum.

When R10 antiserum against N16 type 1 fimbrial immunoprecitins or R2P against N16 type 2 fimbriae were examined by immunoelectron microscopy against N16 cells, both antisera reacted with epitopes located on the fimbriae (Figure 8; R10 reaction not shown). These antisera were used to make polyclonal rabbit IgG immunoaffinity columns for the purification of both types of fimbriae from the N16 French press supernatant.

Purification of N16 Fimbriae from the French Press Supernatant

In addition to the purification of N16 type 2 fimbriae from a crude sonicate by sequential chromatography first on Bio-Gel A-5m and then on the MAb immunoaffinity column, an attempt was made to purify N16 type 1 fimbriae and type 2 fimbriae directly from a crude French press supernatant by immunoaffinity chromatography on rabbit IgG (RIgG) anti-N16 type 1 fimbriae and rabbit IgG anti-N16 type 2 fimbriae columns, respectively. When a 90 ml aliquot (252 mg protein) was chromatographed on the polyclonal anti-N16 type 2 fimbriae immunoaffinity column, the unbound fraction contained both types of fimbriae, and the bound fraction, desorbed with 7 M urea buffer, contained type 2 fimbriae with perhaps a trace of type 1 fimbriae, as determined by immunodot EIA. Since the unbound fraction still contained plenty of type 2 fimbriae, approximately half of it was rechromatographed and eluted as before. The quantity of bound protein desorbed on each run was so low that it was virtually undetectable by A_{280}; also, the recovery from the second run appeared to be lower than the first, suggesting that the bound antigen was not being desorbed completely with 7 M urea.

Figure 8. Electron micrograph of A. naeslundii N16 cells showing indirect immunogold labeling of type 2 fimbriae. The cells were incubated with R2P antiserum (rabbit anti-N16 type 2 fimbrial immunoarcs) and then with goat anti-rabbit IgG conjugated to colloidal gold (diameter, 10 nm). The gold-labeled cells were prepared for EM by the pseudoreplica technique and were negatively stained with 0.5% uranyl acetate. Bar, 1 μm.

A dissociation experiment was undertaken to assess the efficacy of various buffers in dissociating the antigen-antibody complexes formed between N16 fimbriae and the anti-fimbrial antibodies used to prepare the immunoaffinity columns; the results are summarized in Table 4. Although 8 M urea buffer released 85% of Ag-bound MAb 3B5.A1, it released only 28.8% of rabbit IgG anti-N16 type 1 fimbriae and 18.5% of rabbit IgG anti-N16 type 2 fimbriae. Thus, the 7 M urea buffer normally used as the dissociating buffer for immunoaffinity chromatography would be ineffective at desorbing bound fimbriae from either of the polyclonal rabbit IgG anti-N16 fimbriae immunoaffinity columns. The most effective dissociating buffer for the polyclonal immunoaffinity columns was 6 M NaSCN, which gave 95.4% release of rabbit IgG anti-N16 type 1 fimbriae and 91.0% release for rabbit IgG anti-N16 type 2 fimbriae. There was no evidence that any of the dissociating buffers irreversibly denatured fimbrial epitopes or released fimbriae from the plate during these assays.

Based on the results of the dissociation experiment, the desorbing buffer was changed to 6 M NaSCN, 0.05 M Tris-HCl, pH 7.5, and the rabbit IgG anti-N16 type 2 fimbriae immunoaffinity column was desorbed again to release the fimbriae that were not released by 7 M urea. The bound fractions recovered from the 'type 2' immunoaffinity column were pooled, concentrated, and dialyzed versus TBS by ultrafiltration (Amicon YM10). Crystalline $(NH_4)_2SO_4$ was added to 100% saturation, and the precipitated sample was stored at $4^{o}C$ until further processing.

The unbound material recovered from the rabbit IgG anti-N16 type 2 fimbriae immunoaffinity column was chromatographed on the anti-N16

TABLE 4. Summary of the efficacy of various dissociation buffers in disrupting binding between antibodies to N16 fimbriae and N16 fimbriae coated on Immulon 2 microtiter plates

Dissociation buffer	Antibody samples		
	RIgG α-1	RIgG α-2	MAb 3B5.A1
NaSCN, 6 M	95.4[a]	91.0	85.6
NaSCN, 3 M	47.8	23.4	83.6
NaSCN, 1 M	16.9	6.4	78.0
GuHCl, 6 M	93.0	84.5	ND
GuHCl, 3 M	46.5	30.2	82.2
GuHCl, 1 M	17.1	8.6	76.6
Urea, 8 M	28.8	18.5	85.0
Urea, 4 M	5.3	3.9	73.9
Urea, 2 M	3.1	2.1	33.2
Urea, 1 M	12.1	0.0	7.7
Glycine-HCl, pH 2.5	81.7	64.9	86.7
Glycine-HCl/10% $C_2H_6O_2$ [b], pH 2.5	72.3	54.0	85.6
NH_4OH, pH 11.5	16.8	7.4	82.8
NH_4OH/10% $C_2H_6O_2$, pH 11.5	26.8	10.8	73.6

[a] Percent release of antibody from fimbriae-coated plates.

[b] Ethylene glycol.

type 1 fimbriae immunoaffinity column. Since the column did not bind all the type 1 fimbriae on the first pass, the unbound fraction was rechromatographed for a total of three runs. The bound fractions were eluted with 6 M NaSCN, pooled, concentrated, and dialyzed versus saline by ultrafiltration. Examination of the unbound and bound fractions by immunodot EIA revealed that rabbit Ig was leaching from the column during desorption of the bound fimbriae with 6 M NaSCN. Crystalline $(NH_4)_2SO_4$ was added to the unbound and bound fractions, and they were stored as 75% and 100% saturated solutions at $4^{o}C$.

Fimbrial samples from the French press supernatant, as well as samples obtained from the Batch A and Batch B sonicates, were examined by SDS-PAGE and immunoblot. However, before one can understand the results of those experiments, an explanation of the nature of Actinomyces fimbriae and the patterns they exhibit on immunoblots is essential.

Effects of Various Physical and Chemical Treatments on N16 Fimbriae

The fimbriae of E. coli and other gram-negative bacteria are polymers of smaller subunits, and under the appropriate conditions, they can be dissociated into their constituent monomers. The following experiments were undertaken to (a) determine whether or not N16 fimbriae had a subunit architecture or exhibited other properties similar to those reported for the fimbriae of E. coli, (b) provide additional evidence that N16 type 1 fimbriae differed from type 2 fimbriae, (c) see whether certain procedures caused changes in the molecular weight or antigenicity of fimbrial bands.

Aliquots of the N16 crude French press sample were subjected to a variety of physical and chemical treatments. The first was addition of $MgCl_2$. Unlike E. coli fimbriae (83), N16 fimbriae did not precipitate with a final concentration of 0.1 M $MgCl_2$.

Since the N16 crude French press supernatant contained both types of fimbriae, as well as non-fimbrial components, the effects of the other treatments on each type of fimbriae were demonstrated by SDS-PAGE immunoblot analysis, using antibodies monospecific for type 1 or type 2 fimbriae. For some of these experiments a convenient method for exchanging buffers and concentrating samples was needed. Acetone precipitation appeared to be the ideal choice, but first it had to be established that acetone precipitation would give total recovery of fimbriae without adversely affecting their immunological reactivity. When acetone precipitates of the crude fimbrial sample were compared to the original sample, they gave identical patterns on immunoblots. Acetone precipitation was thus employed whenever necessary to accomplish the objectives cited above.

The effects of some of the physical and chemical treatments on N16 type 1 and type 2 fimbriae are shown in Figures 9 and 10, respectively. Exposure to Freon appeared to have no effect on N16 fimbriae; therefore, if extensive lysis were to occur during sonication or French press shearing of cells to remove fimbriae, lipids could be extracted from the crude fimbrial sample with Freon before applying it to gel filtration or immunoaffinity columns.

Treatment with mutanolysin or lysozyme (lysozyme not shown) did not change the fimbrial patterns observed on immunoblots. This suggested that the ladderlike series of bands >100 kd represented

Figure 9. Immunoblot analysis of the effects of various physical and chemical treatments on N16 type 1 fimbriae. Aliquots of the N16 antigen sample were treated as indicated, then prepared for SDS-PAGE by heating at 100 C for 5 min without reduction. Each lane was loaded with 1 µg of total protein, based on the protein concentration prior to treatment. Prestained molecular weight standards were mixed with two of the samples; their positions on the blot were marked with a ballpoint pen, and approximate molecular weights are expressed in kilodaltons. The blot was developed with R10 IgG (anti-N16 type 1 fimbriae) at 1 µg per ml as the primary antibody.

Periodate-oxidized
Untreated
Lancefield-extracted
0.1 N HCl, 37 C, 1 h
0.1 N NaOH, 37 C, 1 h
8 M urea
Mutanolysin
Untreated
0.1 N HCl, 100 C, 5 min
0.1 N HCl, 100 C, 5 min
Untreated
Freon-extracted
Sonicated 10 min
Sonicated 5 min
Sonicated 1 min
100 C, 60 min
100 C, 5 min
65 C, 30 min
37 C, 60 min
200 -
97 -
68 -
43 -
26 -
18 -
14 -

Figure 10. Immunoblot analysis of the effects of various physical and chemical treatments on N16 type 2 fimbriae. This experiment was identical to that described in Figure 9, except that the blot was developed with R2P IgG (anti-N16 type 2 fimbriae) as the primary antibody, and the lane order of the samples is reversed.

37 C, 60 min
65 C, 30 min
100 C, 5 min
100 C, 60 min
Sonicated 1 min
Sonicated 5 min
Sonicated 10 min
Freon-extracted
Untreated
0.1 N HCl, 100 C, 5 min
0.1 N HCl, 100 C, 5 min
Untreated
Mutanolysin
8 M urea
0.1 N NaOH, 37 C, 1 h
0.1 N HCl, 37 C, 1 h
Lancefield-extracted
Untreated
Periodate-oxidized
200
97
68
43
26
18
14

fimbriae of different lengths rather than a fimbrial protein attached to different lengths of peptidoglycan.

Sonication generated some fragments that were not evident in the untreated control. The longer the sample was sonicated, the more fragments were generated and the greater the intensity of the immunological reaction. However, the generation of different molecular weight bands by sonication did not appear to be completely random. The highest molecular weight material was broken down into smaller fragments, but the breakdown products generally were the same molecular weight as fragments already present in the untreated control. The fimbriae were fairly resistant to breakage into fragments smaller than 100 kd; after 10 min of continuous sonication, most of the fimbrial bands were still greater than 100 kd, even though the most immunodominant bands were in the 35-65 kd range.

N16 fimbriae were not affected by 8 M urea or saturated guanidine hydrochloride, treatments that would dissociate some types of E. coli fimbriae into subunit monomers (61,62).

Another method that has been reported to disaggregate E. coli fimbriae, i.e. 0.1 N HCl at 100°C for 5 min (31), did not have a similar effect on N16 fimbriae. Instead of causing dissociation into monomers, hot acid caused loss of band resolution, probably as a result of acid hydrolysis. This also occurred with Lancefield extraction (0.04 N HCl at 100°C for 15 min). However, 0.1 N HCl at 37°C for 1 h had little, if any, effect on N16 fimbriae; and heating at 100°C in the absence of a pH<2 did not cause loss of band resolution. So, it was the combination of a very acidic pH and 100°C that was destructive to the N16 fimbriae.

Base was even more destructive than acid, as judged by the total loss of band resolution and substantially reduced antigenicity when N16 fimbriae were incubated with 0.1 N NaOH at 37°C for 1 h.

Sodium periodate oxidation also caused some loss of band resolution but not to the extent seen with acid or base hydrolysis.

There was some loss of antigenicity with increasing temperature and, as might be expected, heating at 100°C for 1 h was the most destructive.

N16 type 1 fimbriae could be distinguished from type 2 fimbriae by their different patterns on immunoblots, i.e. the different molecular weight distribution of the unreduced immunodominant bands. Disregarding their intrinsically different patterns, the two fimbrial types generally behaved similarly in response to the various treatments. For example, neither was affected by high concentrations of urea or guanidine HCl, but both were most adversely affected by incubation with 0.1 N NaOH. The decrease in antigenicity of N16 fimbriae when incubated with NaOH or heated at 100°C for 60 min is consistent with the findings of Masuda et al. (77) for A. viscosus WVU627 fimbriae. Although they reported that WVU627 fimbriae were also labile when incubated with 0.1 N HCl at 37°C for 60 min, a similar effect was not observed with N16 fimbriae. However, immunoblot analysis of SDS-PAGE-resolved proteins may not have been the best way to examine the effects of the various physical and chemical treatments because denaturation of the proteins by heating in SDS-PAGE sample buffer may mask the true effects of the other treatments. It might have been better to use an assay that could quantitate the amount of immunoreactive fimbriae remaining after

treatment, perhaps by using LRI, XIEP, EIA, or radioimmuno-precipitation. On the other hand, SDS-PAGE immunoblots were probably the best way to see the effects of sonication.

A. naeslundii N16 fimbriae were not disaggregated by any of the methods that would dissociate E. coli fimbriae, pili, or flagella (31,61,62,70,83). These results are consistent with the observation that A. viscosus T14V fimbriae cannot be completely dissociated either (12,84).

Immunoblots were also used to assess the effects of various proteases on the molecular weight distribution and immunological reactivity of N16 type 1 and type 2 fimbrial bands. All the proteases eliminated the immunoreactive bands >200 kd for both types of fimbriae. Samples treated with papain, Pronase, or proteinase K exhibited a total loss of immunoreactive type 1 and type 2 fimbrial bands. However, since papain caused only limited digestion of the BSA control as determined from the presence of multiple bands in the range below 35 kd on a silver-stained SDS-PAGE gel, it is possible that papain and perhaps the other enzymes destroyed the fimbrial epitopes without completely digesting the fimbriae.

The effects of chymotrypsin, trypsin, and V8 protease on N16 type 1 and type 2 fimbriae are shown in Figure 11 and Figure 12, respectively. Each of these enzymes caused limited digestion of both types of fimbriae, but the resulting immunoblot patterns were different for each type of fimbriae. Trypsin caused a total loss of band resolution for type 1 fimbriae; except for two discrete bands at <14 kd, trypsin-treated type 1 fimbriae gave an immunoreactive smear from 40-200 kd on immunoblot. On the other hand, the immunoreactive

Figure 11. Immunoblot analysis of the effects of digestions with different proteases on N16 type 1 fimbriae. The lanes containing untreated or enzyme-treated N16 antigens were loaded with 2 μg of N16 protein, based on the concentration of the N16 sample prior to treatment, whereas the lanes containing enzyme only (lanes 3, 5, and 8 from left to right) were loaded with quantities of enzyme equivalent to those in the enzyme-treated samples. All samples were unreduced, except for the one marked R. Prestained molecular weight standards were mixed with the untreated control in lane 1. The blot was developed with R10 IgG (anti-N16 type 1 fimbriae) at 2.5 μg per ml as the primary antibody.

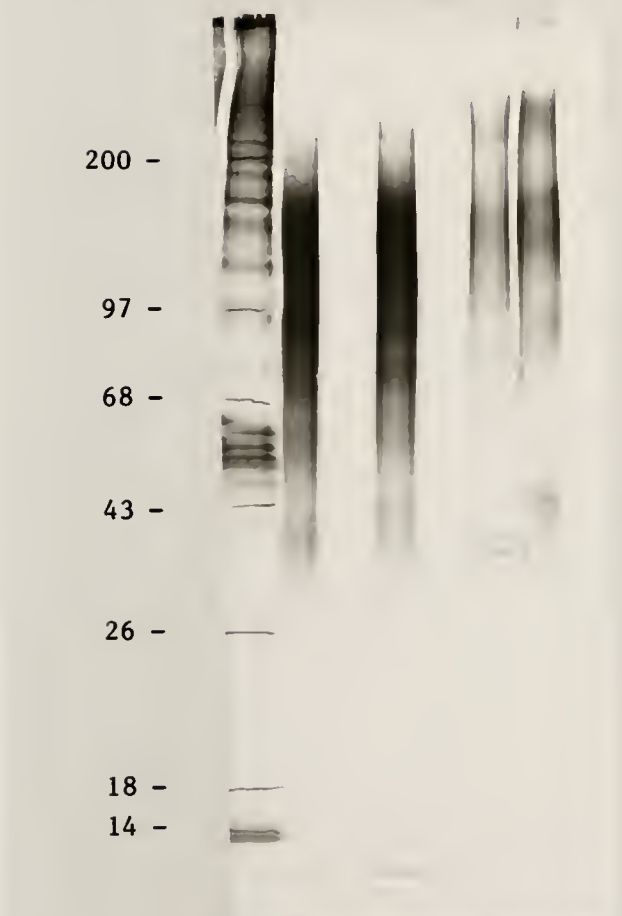
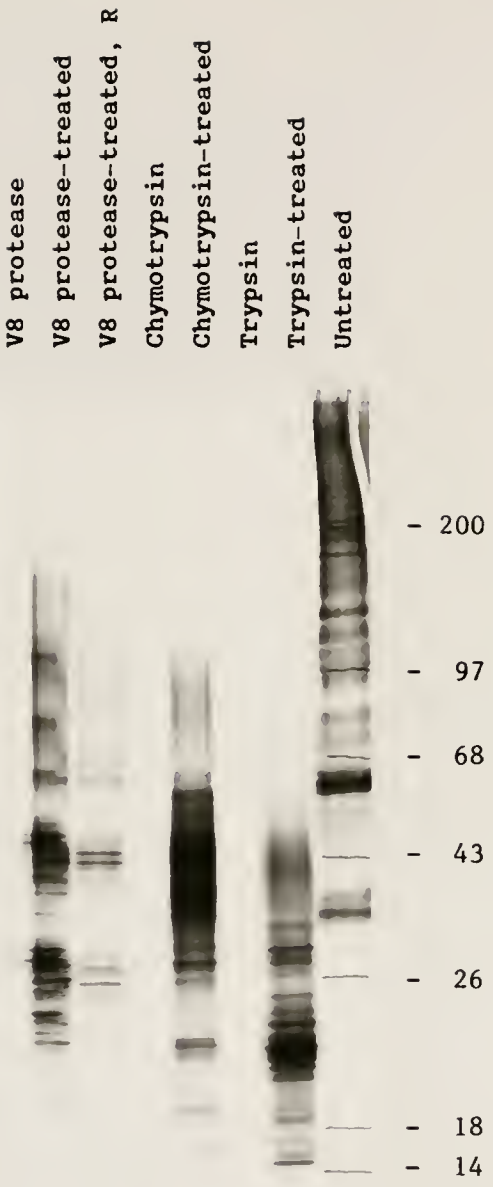

Untreated
Trypsin-treated
Trypsin
Chymotrypsin-treated
Chymotrypsin
V8 protease-treated, R
V8 protease-treated
V8 protease
200 -
97 -
68 -
43 -
26 -
18 -
14 -

Figure 12. Immunoblot analysis of the effects of digestions with different proteases on N16 type 2 fimbriae. This experiment was identical to that described in Figure 11, except that the blot was developed with R2P IgG (anti-N16 type 2 fimbriae) as the primary antibody, and the lane order reads right to left.


V8 protease
V8 protease-treated
V8 protease-treated, R
Chymotrypsin
Chymotrypsin-treated
Trypsin
Trypsin-treated
Untreated
200
97
68
43
26
18
14

type 2 fimbrial bands after trypsin treatment were mostly between the 14 and 43 kd markers, and for the most part, the bands were discrete rather than unfocused. The effect of chymotrypsin on type 1 fimbriae was very similar to that of trypsin, whereas for type 2 fimbriae, the pattern obtained by digestion with chymotrypsin was quite different from that obtained with trypsin. V8 protease-treated samples were less immunoreactive than the trypsin- or chymotrypsin-treated samples, even though V8 protease appeared to be less efficient than the other two enzymes at digesting the higher molecular weight fragments.

Effects of Temperature and Reduction on N16 Fimbriae

On immunoblots the most immunoreactive N16 type 1 and type 2 fimbrial bands undergo a shift in apparent molecular weight in response to increases in the temperature for SDS-PAGE sample preparation, as demonstrated in Figure 13. In unreduced samples heated at 37°C for 30 min there are a series of type 1 immunoreactive fimbrial bands at or slightly below the 43 kd molecular weight marker, whereas after heating at 100°C for 5 min, the immunodominant bands are in the 57-65 kd range. Both sets of bands are present in the sample heated at 65°C for 15 min, so the immunoblot pattern at this intermediate temperature shows the "transitional state" of proteins switching from their lower molecular weights at 37°C to higher molecular weights at 100°C. Reduction with 0.01 M DTT (or 1% 2-mercaptoethanol) further simplifies the pattern of bands so that with samples reduced at 100°C the primary type 1 fimbrial bands on immunoblots are as follows: the uppermost band has an apparent molecular weight of about 65 kd; there is a broad area of immunoreactivity in the 57-60 kd range, which represents at

Figure 13. Immunoblot analysis of the effects of temperature and reduction on the type 1 and type 2 fimbriae of A. naeslundii N16. Aliquots of the N16 antigen sample were treated with SDS-PAGE sample buffer, with or without 0.1 M dithiothreitol (DTT), by heating at the temperature and times indicated. Each lane of the SDS-PAGE gel was loaded with 1 µg of protein. Approximate molecular weights are expressed in kilodaltons. The left half of the blot was developed with R10 IgG at 5 µg/ml, and the right half was developed with R2P IgG at 5 µg/ml.

Anti-Type 1
Anti-Type 2
37 C, 30 min, - DTT
37 C, 30 min, + DTT
65 C, 15 min, - DTT
65 C, 15 min, + DTT
100 C, 5 min, - DTT
100 C, 5 min, + DTT
100 C, 5 min, + DTT
100 C, 5 min, - DTT
65 C, 15 min, + DTT
65 C, 15 min, - DTT
37 C, 30 min, + DTT
37 C, 30 min, - DTT
- 200
- 97
- 68
- 43
- 26
- 18
- 14

least two bands, including a component at 57 kd that reacts with normal RIgG; then there is a weaker doublet at about 53-54 kd.

In unreduced samples heated at 37°C, the immunoblot pattern for type 2 fimbrial bands shows a series of bands near the 43 kd marker, whereas unreduced samples heated at 100°C show a very reactive doublet at about 62 and 63 kd and several closely spaced bands at about 39-40 kd. As with the type 1 pattern, the 65°C type 2 pattern showed the transition from the 37°C to the 100°C pattern. Type 2 fimbriae were also affected by reduction in that the prominent doublet at 62-63 kd in the nonreduced sample is replaced by a single 63 kd band, and a minor doublet is still evident at 39-40 kd. On type 2 blots there was a very weakly immunoreactive band at about 23 kd that was present in reduced and nonreduced samples prepared at any temperature. A similar band at 18 kd (not visible in Figure 13) was sometimes visible on type 1 blots. These low molecular weight bands might represent fimbrial subunits (E. coli fimbrial subunits are approximately this size); however, they may just be products of degradation.

The observation that A. naeslundii N16 fimbrial proteins are heat modifiable is consistent with the report of Yeung et al. (108) that A. viscosus T14V type 1 fimbriae have a heat modifiable subunit with an apparent molecular weight of about 50 kd at 37°C or 65 kd at 100 C. Similarly, Yeung et al. reported that the fimbrial subunit of A. naeslundii WVU45 type 2 fimbriae has an apparent molecular weight of about 48 kd at 37°C or 60 kd at 100°C (109, M. K. Yeung, personal communication).

It is likely that the observed shifts in molecular weights of fimbrial proteins in response to heat are the result of changes in

conformation. Strong intramolecular interactions at the lower temperature may prevent complete unfolding of the protein and saturation with SDS, thereby altering the mobility in gels. The results obtained with the cloned T14V type 1 fimbrial subunit (108) tend to support such an explanation. Intermolecular interactions of a noncovalent or covalent nature could also prevent complete denaturation of fimbrial complexes and account for some of the observed differences in the 37°C and 100°C immunoblot patterns.

The observation that N16 fimbriae were susceptible to reducing agents differs from all previous reports on fimbriae from other strains of Actinomyces (76,77,108). However, Masuda et al. (76) were unable to get many of their fimbrial samples to migrate into 5 or 7% SDS-PAGE gels, and their single percentage gels would not have the resolving power of the 5-20% gradient gels used in this study. Thus, solubility problems and inadequate resolution of proteins with very similar molecular weights might have prevented them from detecting the effects of reduction on fimbrial bands. This is especially likely in light of the finding of Yeung et al. (108) that the cloned T14V type 1 fimbrial subunit migrated as a single band in 10 or 12% gels but as a doublet in 5-12% gradient gels.

If one were examining the effect of reduction on a purified protein complex that migrated as a single band on SDS-PAGE gels, one would expect that a reduction would cause a single higher molecular weight band to dissociate into one or more lower molecular weight bands, depending on whether the complex was composed of identical or dissimilar polypeptide chains held together by disulfide bonds. However, fimbrial immunoblot patterns are too complex to allow such a

simple interpretation. A comparison of the differences between the patterns of type 2 fimbriae prepared with or without reduction at 100°C indicates that reduction caused changes in the visible bands at virtually every molecular weight level. There are too many bands present to speculate on whether any particular band was derived from another, but the fact that the reduced samples appear to have fewer and better focused bands than nonreduced samples suggests that the reduction of intra- or intermolecular disulfide bonds allows complete denaturation and SDS saturation of the proteins, thereby overcoming conformational variations that affect the mobilities and apparent molecular weights of fimbrial fragments.

Another possible explanation for the presence of fewer reactive bands in reduced samples is that reduction may destroy the fimbrial epitopes on some bands so that they are no longer visible on immunoblots. This explanation would be more plausible if the blots were developed with monoclonal antibody; but since they were developed with polyclonal antibodies, it is not likely that destruction of epitopes could explain these results.

In summary, since complete dissociation of N16 fimbriae was not possible, immunoblot patterns exhibited a multitude of fimbrial bands. The simplest pattern of bands was obtained with fimbrial samples heated at 100°C in the presence of DTT or 2-mercaptoethanol. In addition to the ladderlike series of bands in the high molecular weight range, there were several strongly immunoreactive bands (referred to as fimbrial subunits) in the 35-65 kd range. A. viscosus T14V type 1 and type 2 fimbriae have been reported to give immunoblot patterns (32,108) similar to those

obtained in this study with N16 fimbriae. The fimbrial subunit patterns for unreduced and reduced A. naeslundii N16 type 1 and type 2 fimbriae are summarized in Figure 14.

Assessment of the Purity of Fimbrial Samples by SDS-PAGE-Immunoblot

When aliquots of the type 1 and type 2 fimbriae purified from the polyclonal antibody immunoaffinity columns were examined by SDS-PAGE and immunoblot, it was apparent from the presence of immunoglobulin heavy and light chains on the silver-stained SDS-PAGE gel (not shown) that both samples were contaminated with RIgG that had leached off the immunoaffinity columns. Consequently, immunoblot analysis of these two fractions was complicated by the reaction of the peroxidase conjugate with RIgG present in the fimbrial samples. The immunoblot patterns of these two samples as well as those for the crude French press supernatant, Batch A A-5m peak 1, Batch B 35% saturated $(NH_4)_2SO_4$ precipitate of the crude sonicate, and two samples of the MAb-purified type 2 fimbriae, are shown in Figure 15.

The fimbrial subunits for both type 1 and type 2 fimbriae appear to be present in all the samples except the two MAb-purified fractions, although the weak type 1 bands in the type 2 fimbriae purified from the RIgG column may actually be a reaction of conjugate with immunoglobulin heavy chains contaminating the preparation. The 65 kd type 1 fimbrial subunit is readily apparent only in the type 1 fimbriae recovered from the RIgG column, indicating that this fraction was enriched for type 1 fimbriae. That band is also present, but not as obvious, in the French press supernatant, sonicate, and A-5m peak 1 samples, all of which should contain both types of fimbriae. The 63 kd type 2 fimbrial subunit is present in the type 2 fimbrial samples,

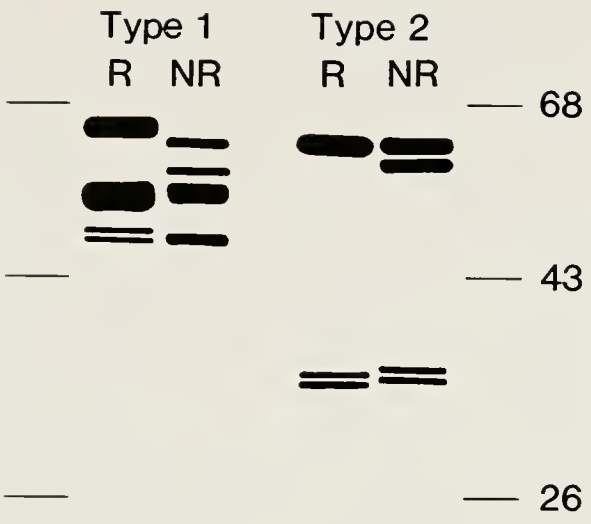
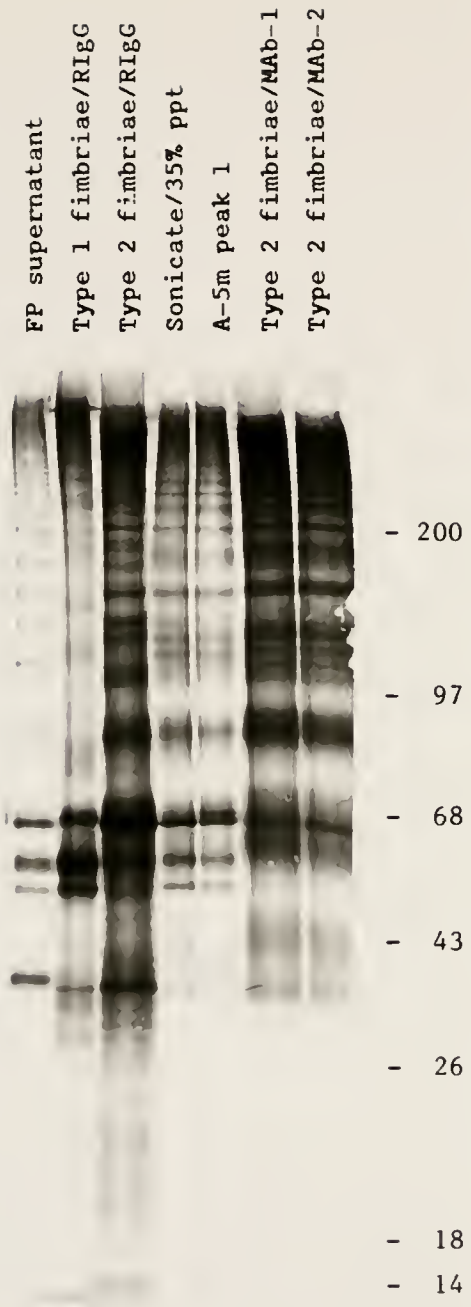


Figure 14. Line drawing summarizing the "fimbrial subunit" patterns obtained on immunoblots for A. naeslundii N16 type 1 and type 2 fimbriae for samples heated at 100 C for 5 min. R = reduced with 0.01 M DTT; NR = unreduced. Approximate molecular weights are expressed in kilodaltons.

Figure 15. Immunoblot analysis of several preparations of N16 fimbriae at different stages of purification. Crude French press (FP) supernatant was the starting material for immunoaffinity purification of type 1 and type 2 fimbriae from the RIgG anti-type 1 and anti-type 2 columns, respectively. The middle lane contains the Batch B 35% $(NH_4)_2SO_4$ precipitate of the crude 2 min sonicate. Also shown are A-5m peak 1 from Batch A and two samples of type 2 fimbriae purified on the MAb 3B5.A1 column. All samples were reduced with DTT (0.01 M final concentration). The blot was developed with a combination of R10 IgG at 5 µg/ml and R2P IgG at 2.5 µg/ml, so both types of fimbriae are evident.

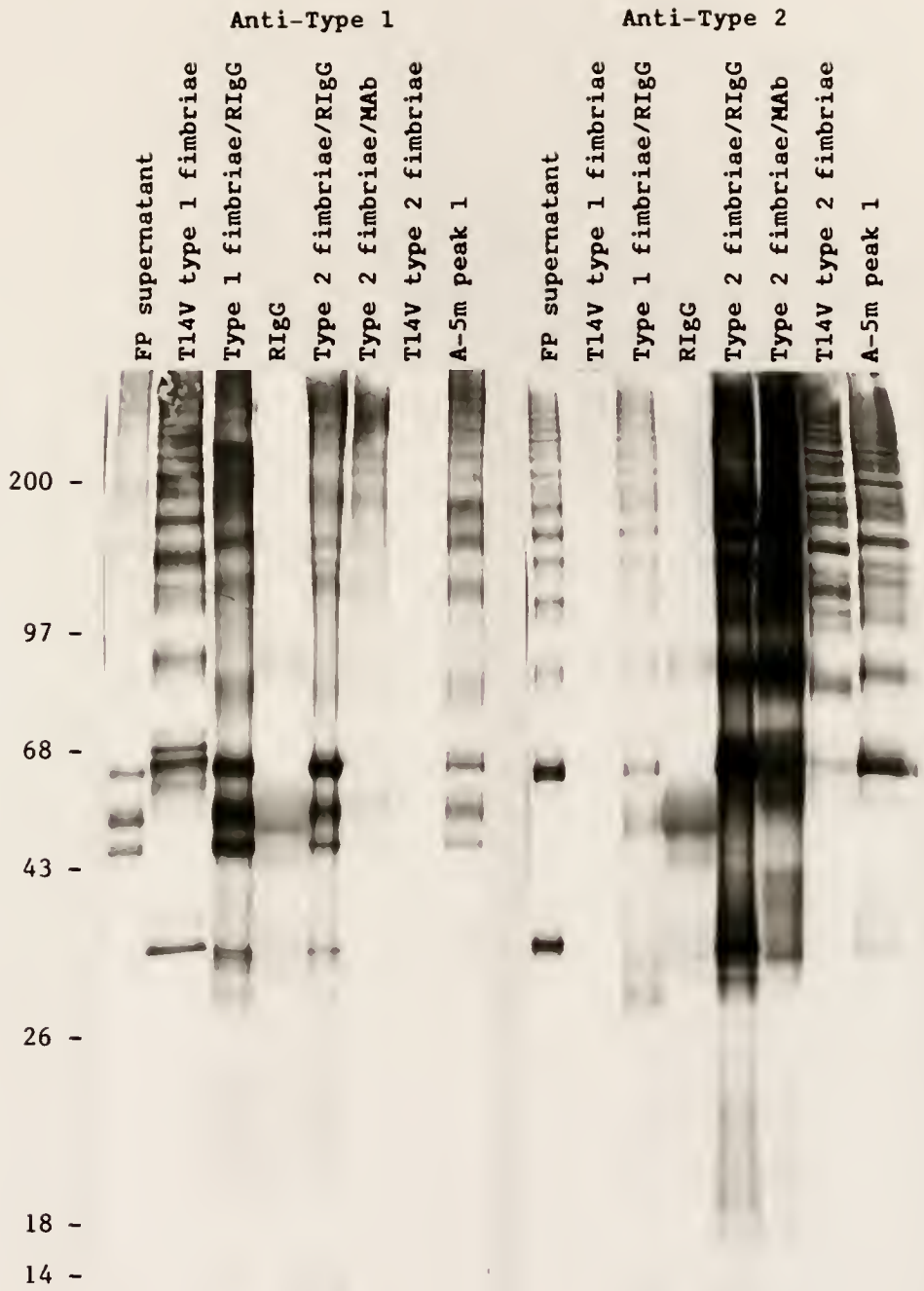

FP supernatant
Type 1 fimbriae/RIgG
Type 2 fimbriae/RIgG
Sonicate/35% ppt
A-5m peak 1
Type 2 fimbriae/MAb-1
Type 2 fimbriae/MAb-2
200
97
68
43
26
18
14

but the 39-40 kd doublet is not apparent. In fact, the 39-40 kd doublet was present only in the crude French press supernatant, so there is some question as to whether or not it is a type 2 fimbrial subunit.

An attempt was made to remove the RIgG contaminating the immunoaffinity purified type 1 and type 2 samples by adsorption with protein A-Sepharose, but SDS-PAGE analysis of the samples after protein A adsorption indicated that immunoglobulin chains were still present in the fimbrial samples.

The protein A-adsorbed fimbrial samples and several of the other samples were examined again by immunoblot analysis as shown in Figure 16. Duplicate blots were developed with either R10 against N16 type 1 fimbriae or R2P against N16 type 2 fimbriae. Also included for comparison to N16 fimbriae were samples of purified T14V type 1 and type 2 fimbriae. The T14V fimbrial samples appeared to be pure since type 2 fimbriae did not react with RIgG against N16 type 1 fimbriae or vice versa. On the other hand, the N16 type 2 fimbriae recovered from the MAb immunoaffinity column exhibited minor contamination with type 1 fimbriae, as determined previously by XIEP (Figure 6C). The subunit pattern for T14V type 1 fimbriae on the immunoblot developed with RIgG against N16 type 1 fimbriae differs from the N16 type 1 fimbrial subunit pattern, whereas the type 2 patterns for each appear to be more similar. Another interesting observation is that the 63 kd N16 type 2 subunit (in the French press supernatant reacted with anti-type 2) appears as a doublet. On all the other immunoblots it appeared to be a single band, but this sample was electrophoresed longer than usual, and better resolution was obtained.

Figure 16. Immunoblot analysis of A. naeslundii N16 and A. viscosus T14V fimbrial samples. All samples were reduced with DTT at 100 C. The left half of the blot was developed with R10 IgG anti-N16 type 1 fimbriae, and the right half was developed with R2P IgG anti-N16 type 2 fimbriae.


Anti-Type 1
Anti-Type 2
FP supernatant
T14V type 1 fimbriae
Type 1 fimbriae/RIgG
RIgG
Type 2 fimbriae/RIgG
Type 2 fimbriae/MAb
T14V type 2 fimbriae
A-5m peak 1
200
97
68
43
26
18
14

In summary, the N16 fimbrial samples obtained by immunoaffinity chromatography were enriched for one of the fimbrial types, but none of the samples were completely pure. The MAb immunoaffinity column appears to offer the most promise for purification of type 2 fimbriae, but it might be necessary to incorporate a detergent into the starting buffer to prevent nonspecific adsorption of type 1 fimbriae. As for the RIgG columns, it was probably too much to expect that they could produce pure fimbriae from totally crude starting material. However, since they required such harsh conditions to elute bound fimbriae that some RIgG also was stripped from the columns, it is unlikely that these immunoaffinity columns would be as useful as MAb columns.

Antigenic Relatedness of Actinomyces Fimbriae

Ouchterlony Analysis

Like the type 1 and type 2 fimbriae of A. viscosus T14V (20), the type 1 and type 2 fimbriae of A. naeslundii N16 were found to be unrelated antigenically, as demonstrated by the reactions of non-identity between type 1 and type 2 fimbrial precipitins in immunodiffusion (Figure 17A). A reaction of partial identity between the type 2 fimbrial bands precipitated by MAb 3B5.A1 and the polyclonal reference antiserum (Figure 17B) provided additional evidence that MAb 3B5.A1 is specific for an epitope on the type 2 fimbriae; however, the presence of a spur suggests that there are other epitopes on N16 type 2 fimbriae that elicit the formation of Abs in a polyclonal response to the antigen. MAbs 2A3.B3 and 2B5.B6 did not precipitate either type of fimbriae, perhaps because (a) the proportions of antigen and antibody were not at equivalence or (b) the

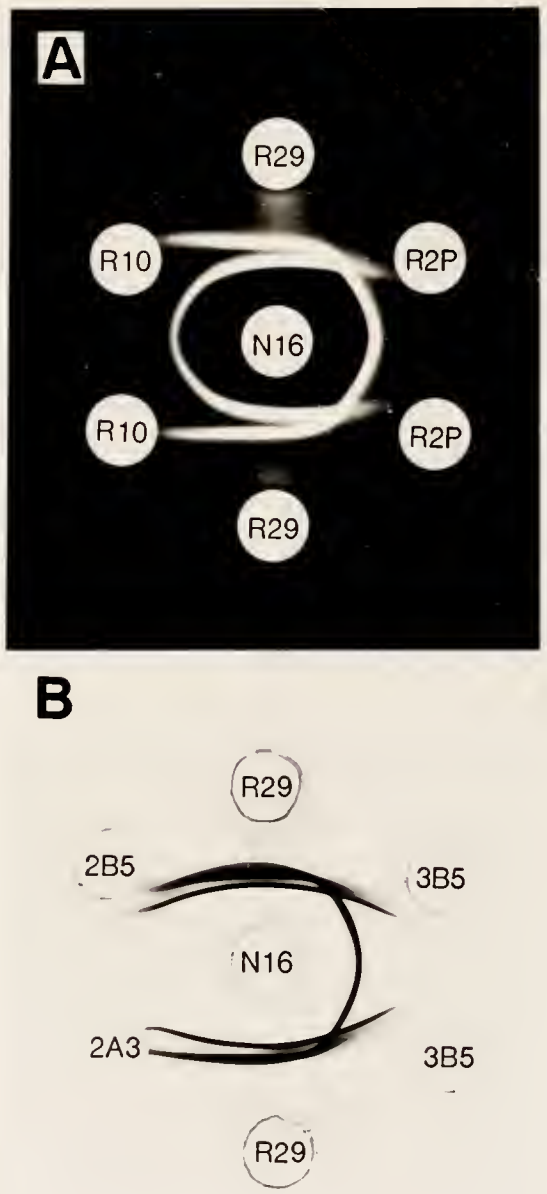


Figure 17. Immunodiffusion reactions of antibodies to N16 fimbriae. The center wells contained N16 fimbriae partially purified from a French press supernatant by precipitation at 20% (w/v) $(NH_4)_2SO_4$. Rabbit antiserum R29 against N16 whole cells precipitated both type 1 and type 2 fimbriae.
A) A reaction of non-identity between type 1 and type 2 fimbriae. Antisera were R10 against N16 type 1 fimbriae and R2P against N16 type 2 fimbriae.
B) A reaction of partial identity between the type 2 fimbrial bands precipitated by MAb 3B5.A1 and the polyclonal reference antiserum R29. MAb 2A3.B3 and MAb 2B5.B6 did not precipitate the fimbriae.

density of fimbrial epitopes was too low, as suggested previously by the sparse distribution of gold particles in immunoelectronmicroscopy.

XIEP-A

The antigenic relatedness among fimbriae from different isolates of A. naeslundii and A. viscosus was examined by XIEP-A. Unlabeled homologous antisera raised against whole cell antigens were incorporated into gels to precipitate fimbrial and sometimes non-fimbrial antigens; in addition, an ^{125}I-labeled antibody specific for one type of fimbriae was incorporated into the gel so that autoradiography would reveal which type of fimbriae reacted with the monospecific antibody. Six fimbriae-specific antibodies were examined, and the results of reactions with A. naeslundii antigens are summarized in Table 5.

R1O rabbit IgG anti-N16 type 1 fimbriae reacted strongly with the type 1 fimbriae of all 10 A. naeslundii serotype 3 isolates but not with the type 2 fimbriae (Figure 18). It cross-reacted with the type 1 fimbriae of A. naeslundii serotype 2 W1544, but it did not react with A. naeslundii serotype 1 W826 (WVU45), a serotype that has been reported to lack type 1 fimbriae (17).

R2P rabbit IgG anti-N16 type 2 fimbriae reacted strongly with the type 2 fimbriae of the 10 A. naeslundii serotype 3 isolates, but it was essentially negative for type 1 fimbriae and the fimbriae of other serotypes of A. naeslundii. Heterologous fimbriae were sometimes visible on autoradiographs, but the intensity was so weak that it was difficult to determine whether the reactions were the result of nonspecific binding or very low levels of cross-reactivity; normal rabbit IgG gave similar results.

Table 5. Summary of the reactions of fimbriae-specific ^{125}I-labeled antibodies with Lancefield-extracted type 1 and type 2 fimbriae of A. naeslundii isolates

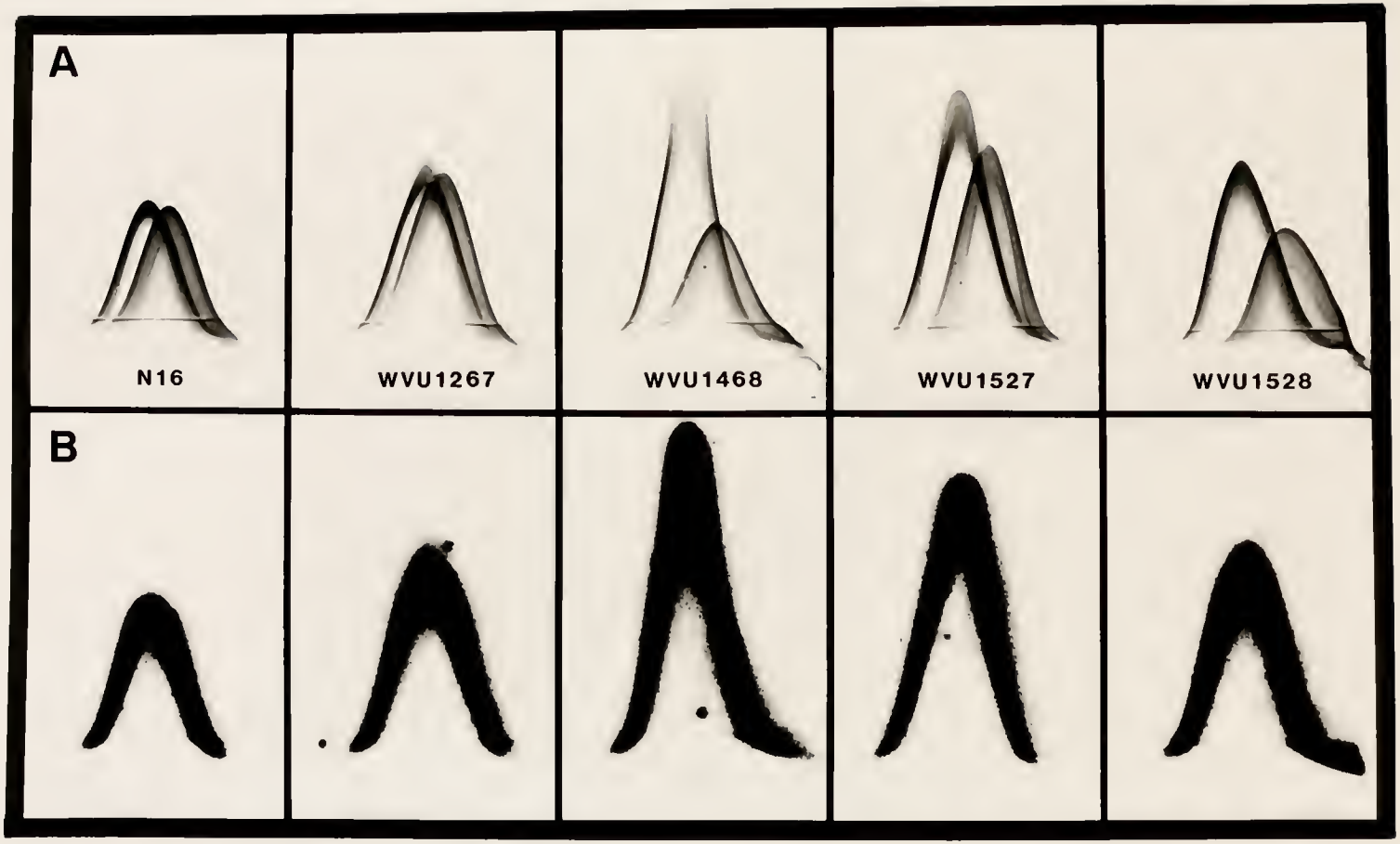
Antigen	^{125}I-Antibody[a]											
	R10		R2P		MAb		R59		R55		R70	
	Type 1	Type 2	Type 1	Type 2	Type 1	Type 2	Type 1	Type 2	Type 1	Type 2	Type 1	Type 2
N16	4[b]	0	0	4	0	4	4	0	1	1	1	1
WVU1267	4	0	0	4	0	4	4	0	1	4	1	2
WVU1468	4	0	0	4	0	4	4	0	1	4	1	2
WVU1527	4	0	0	4	0	4	4	0	1	1	1	1
WVU1528	4	0	0	4	0	4	4	0	1	1	1	1
W1629	4	0	0	4	0	4	3	1-2	1	1	1	2
W2273	4	0	0	4	0	4	4	0	1	1	1	1
W2821	4	0	0	4	0	4	4	0	1	1	1	1
UF92	4	0	0	4	0	4	4	0	1	4	1	1
UF524	2	0	0	4	0	4	4	1	1	1	1	1
W826 (WVU45)	-[c]	0	-	0	-	0	-	0	-	0	-	4
W1544	2-3	0	0	0	0	0	3	1	0	4	0	0

[a] R10 = rabbit IgG anti-N16 type 1 fimbriae
R2P = rabbit IgG anti-N16 type 2 fimbriae
MAb = MAb 3B5.A1 anti-N16 type 2 fimbriae
R59 = rabbit IgG anti-T14V type 1 fimbriae
R55 = rabbit IgG anti-T14V type 2 fimbriae
R70 = rabbit IgG anti-WVU45 type 2 fimbriae

[b] Reactions of ^{125}I-antibodies with fimbrial rockets in XIEP-A were graded on a scale of 0 to 4 relative to the intensity of reaction with the homologous control. The homologous reaction for R59 was 4,1 (type 1, type 2 fimbriae, respectively), and the homologous reaction for R55 was 1,4.

[c] W826 (WVU45) does not have type 1 fimbriae (17).

Figure 18. Reactions of 10 isolates of A. naeslundii serotype 3 with R10 anti-N16 type 1 fimbriae by XIEP-A. All antigens were Lancefield extracts of whole cells. The gel for electrophoresis in the second dimension contained 0.5-0.75% R29 antiserum against N16 cells plus ^{125}I-labeled R10 RIgG.
A) Stained gels demonstrating the presence of type 1 and type 2 fimbriae.
B) Autoradiographs (23.5 h) demonstrating that R10 binds to the type 1 fimbriae of each isolate.


A
N16
WVU1267
WVU1468
WVU1527
WVU1528
B

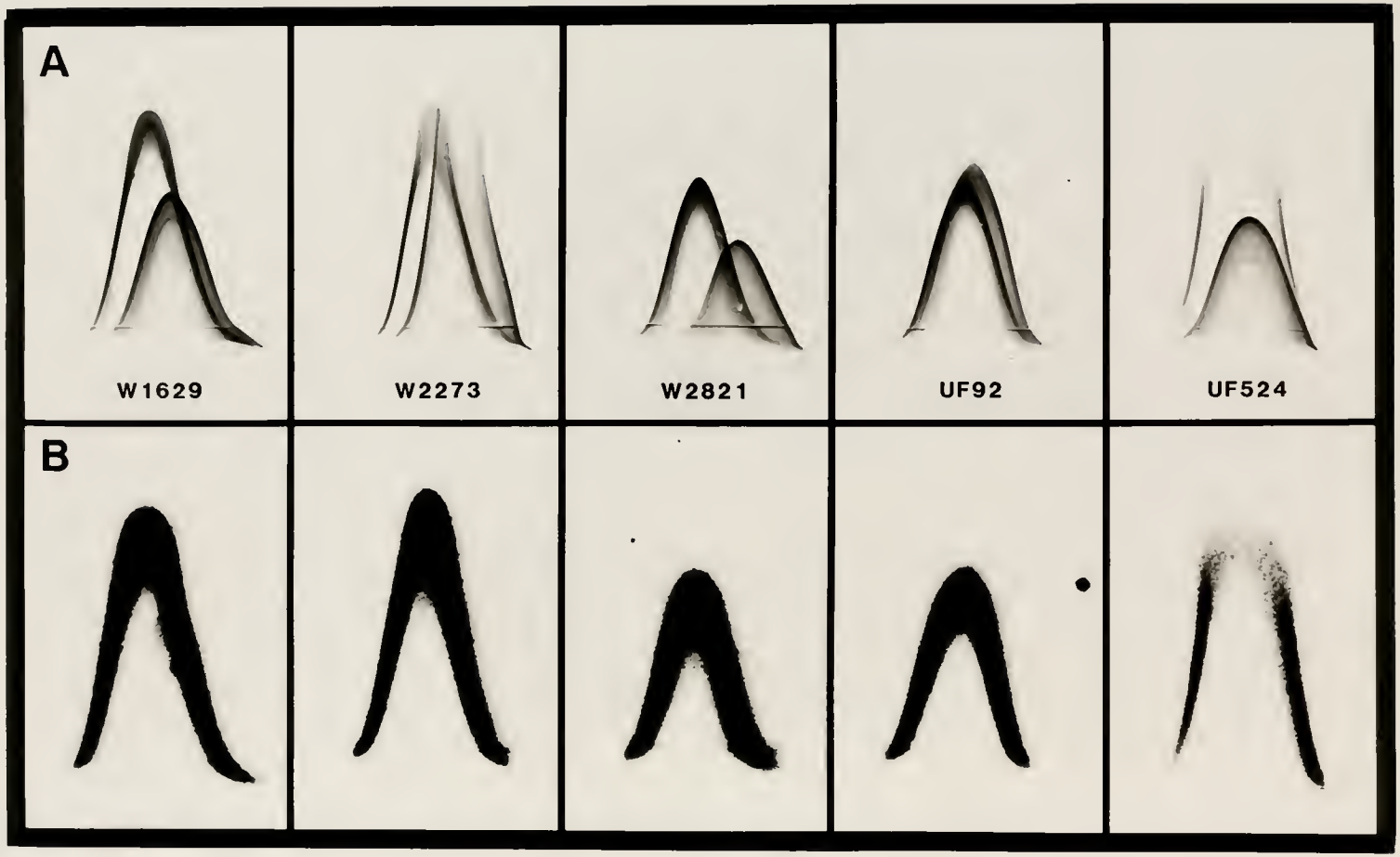


Figure 18. --continued

MAb 3B5.A1 gave the same pattern of reactivity as the polyclonal anti-N16 type 2 fimbriae: it reacted only with the type 2 fimbriae of the A. naeslundii serotype 3 isolates (Figure 19), thereby providing confirmation for the RIA data, which indicated that MAb 3B5.A1 recognizes a serotype-specific epitope.

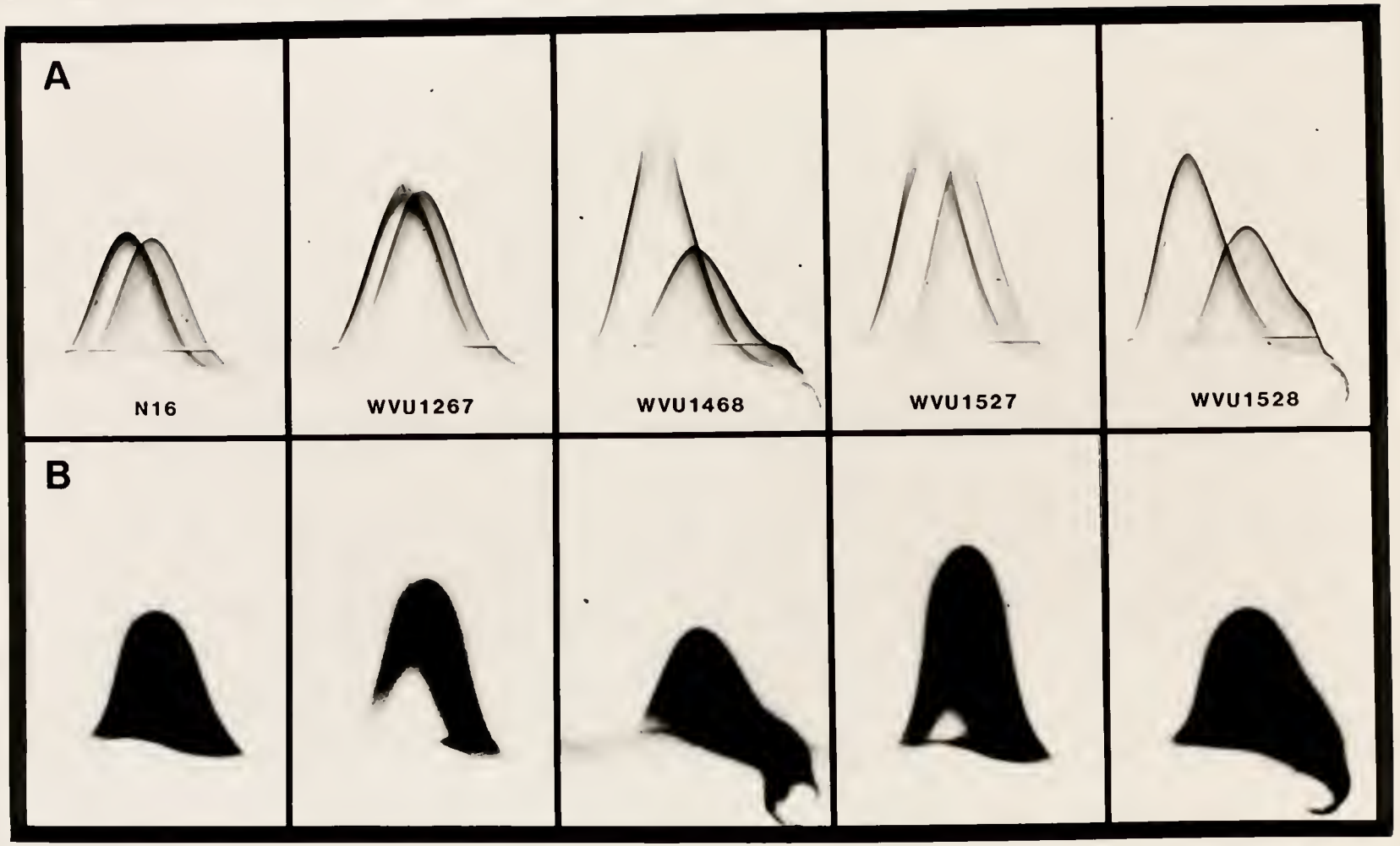
R59 rabbit IgG anti-T14V type 1 fimbriae cross-reacted with the type 1 fimbriae of all 10 A. naeslundii serotype 3 isolates, as well as the isolate representing A. naeslundii serotype 2, W1544.

R55 rabbit IgG anti-T14V type 2 fimbriae exhibited limited reactivity with fimbriae of A. naeslundii isolates. It reacted strongly with the type 2 fimbriae of only 3 of 10 A. naeslundii serotype 3 isolates (WVU1267, WVU1468, and UF92) and also with A. naeslundii serotype 2 W1544 type 2 fimbriae. With the other 7 A. naeslundii serotype 3 strains both fimbrial rockets were visible on the autoradiographs, but the intensity of reaction would be graded no more than 1+ on a 0 to 4+ scale. This antibody did not react with A. naeslundii W826 fimbriae.

R70 rabbit IgG anti-WVU45 type 2 fimbriae gave a very strong reaction only with the type 2 fimbriae of the homologous strain. Both fimbrial rockets were clearly visible on autoradiographs of heterologous isolates, but most could be rated 1+ relative to the 4+ homologous reaction. The type 2 fimbrial rockets of WVU1267, WVU1468, UF524, and W1629 might be graded slightly higher.

Since R70 was produced by immunizing a rabbit with affinity purified type 2 fimbriae from a strain that does not have any type 1 fimbriae (17), the presence of any reaction with type 1 fimbriae, especially one equal in intensity to the reaction with type 2

Figure 19. Reactions of 10 isolates of A. naeslundii serotype 3 with MAb 3B5.A1 anti-N16 type 2 fimbriae by XIEP-A. All antigens were Lancefield extracts of whole cells. The gel for electrophoresis in the second dimension contained 0.5-0.75% R29 antiserum against N16 cells plus ^{125}I-labeled MAb 3B5.A1 IgG.
A) Stained gels demonstrating the presence of type 1 and type 2 fimbriae.
B) Autoradiographs (20 h) demonstrating that MAb 3B5.A1 binds to the type 2 fimbriae of each isolate.


A
N16
WVU1267
WVU1468
WVU1527
WVU1528
B

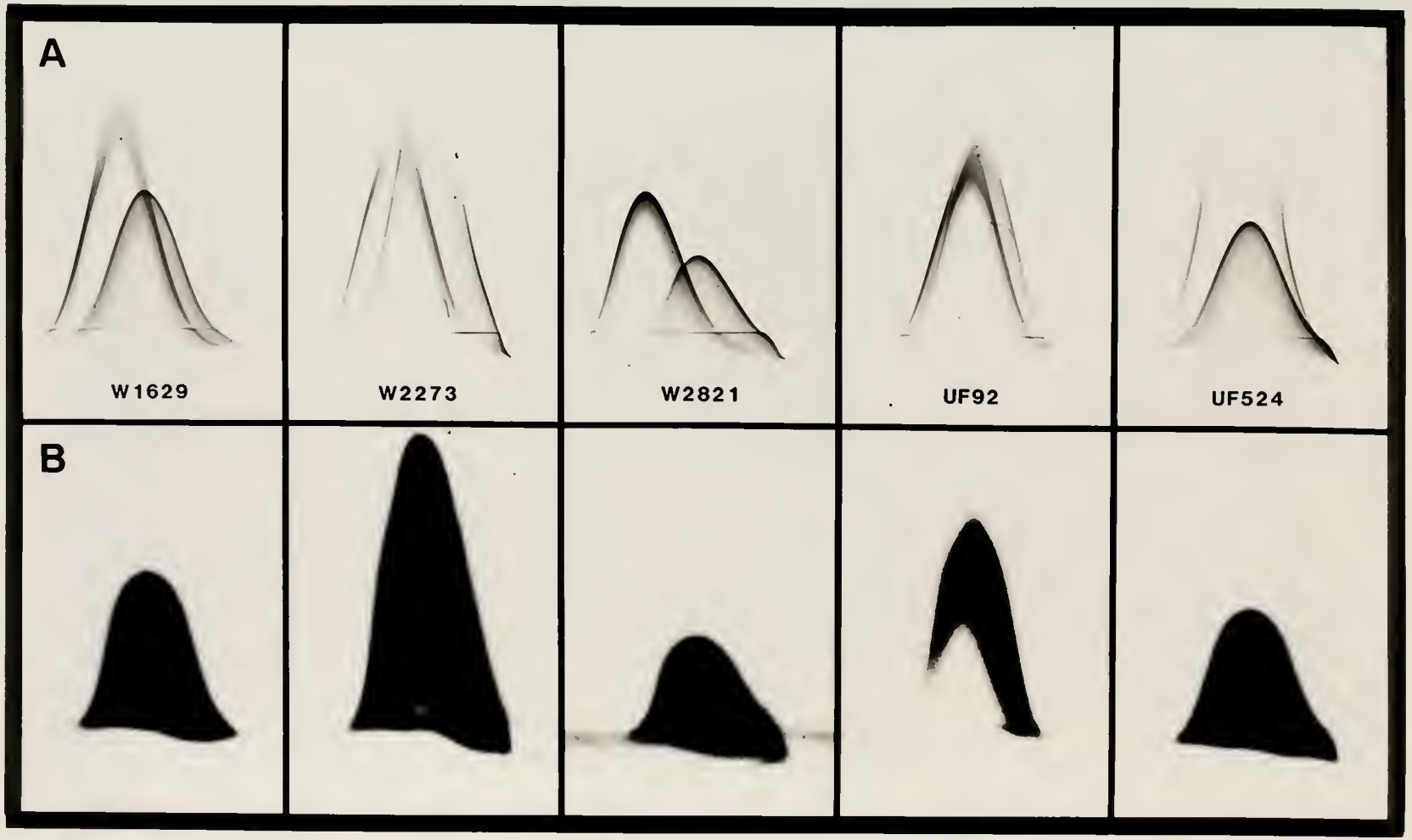


Figure 19. -- continued

fimbriae, was somewhat surprising. However, it points out the problems associated with trying to interpret weak reactions on autoradiographs. In this instance the weak reactions may be a result of the presence of preimmune Abs to fimbriae of other strains; or perhaps the fimbriae used as immunogen were attached to cell wall components that elicited Abs to non-fimbrial determinants present also in Lancefield extracts of cells. When immunoprecipitates of RIgG and fimbriae are used as immunogens, as they were in this study and others (17,92), there is the added possibility of eliciting anti-allotype or anti-idiotype Abs such that ^{125}I-RIgG might bind to the RIgG precipitating the fimbrial Ags. Thus, weak reactions that result from binding of specific Abs to fimbrial epitopes present in low numbers are virtually impossible to distinguish from reactions that would be considered "false positives".

The ability of the six anti-fimbriae antibodies, as well as a normal rabbit IgG control, to react with isolates of A. viscosus serotype 2 was assessed with Lancefield extracts of M100, W1053, and T14V. The Lancefield-extracted antigens of these three strains gave patterns of reactivity in XIEP-A similar to those shown for purified (non-Lancefield extracted) T14V fimbriae (Figure 20). On a scale of 0 to 4+, the reactions with T14V fimbriae were graded as shown in Table 6. The grades were assigned according to the relative intensities of the reactions on the original autoradiographs because the reactions with heterologous ^{125}I-Abs (R10 and R2P) appear to be more intense in the photographs than they were in the original autoradiographs.

Figure 20. Reactions of fimbriae-specific antibodies with A. viscosus T14V fimbriae by XIEP-A. The antigen for each panel was 6.4 µg of purified T14V fimbriae (kindly provided by J.O. Cisar). The stained gels (upper panels) show both types of fimbriae precipitated by UF-1 rabbit antiserum against T14V cells. The 24 h autoradiographs (lower panels) demonstrate the binding of the following ^{125}I-labeled antibodies:

A) R59 anti-T14V type 1 fimbriae;
B) R55 anti-T14V type 2 fimbriae;
C) R10 anti-N16 type 1 fimbriae;
D) R2P anti-N16 type 2 fimbriae.

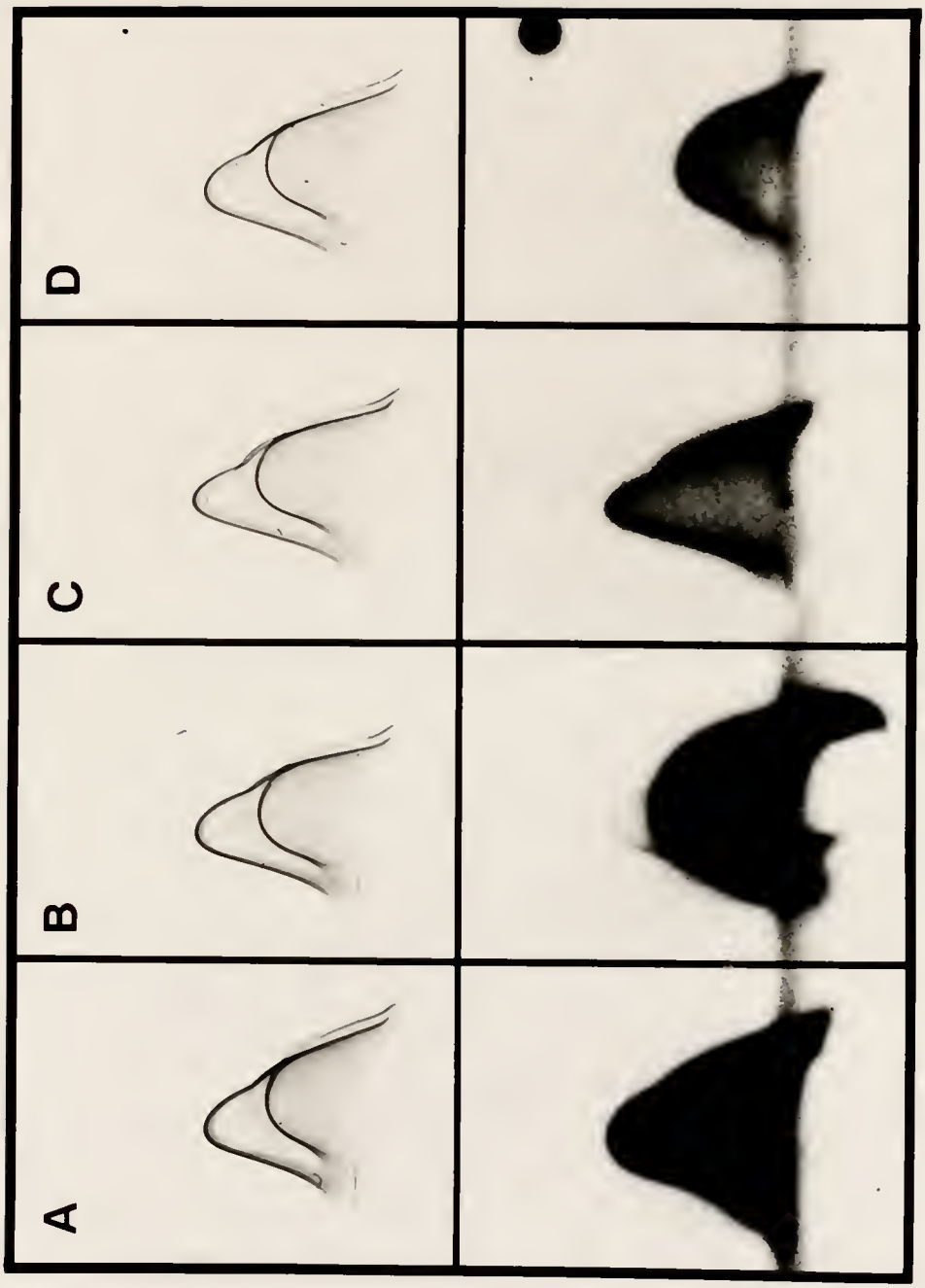

A
B
C
D

TABLE 6. Reactions of fimbriae-specific ^{125}I-labeled antibodies with A. viscosus T14V fimbriae

^{125}I-Antibody	T14V fimbrial antigens[a]	
	Type 1	Type 2
R59 rabbit IgG anti-T14V type 1 fimbriae	4[b]	0
R55 rabbit IgG anti-T14V type 2 fimbriae	0-1	4
R70 rabbit IgG anti-WVU45 type 2 fimbriae	0-1	0-1
R10 rabbit IgG anti-N16 type 1 fimbriae	1-2	0
R2P rabbit IgG anti-N16 type 2 fimbriae	0	1-2
MAb 3B5.A1 anti-N16 type 2 fimbriae	0	0

[a] Sample of purified T14V fimbriae (types 1 and 2) obtained from J.O. Cisar.

[b] Reactions of ^{125}I-antibodies with fimbrial rockets in XIEP-A graded on a scale of 0 - 4+ relative to the intensity of the reactions of normal rabbit IgG and homologous antibodies, respectively.

Polyclonal Abs to N16 type 1 or type 2 fimbriae cross-reacted with the corresponding type of A. viscosus serotype 2 fimbriae, but the reactions were much weaker than the homologous reactions. MAb 3B5.A1 did not react with A. viscosus serotype 2 strains in XIEP-A.

Thus, when examined by XIEP-A, all 10 strains of A. naeslundii serotype 3, one strain of A. naeslundii serotype 2 (W1544), and 3 strains of A. viscosus serotype 2 (T14V, W1053, and M100) had both types of fimbriae as determined by reactions with fimbriae-specific Abs. In every case, the Coomassie-stained patterns revealed that type 1 fimbriae migrated slightly more toward the anode than type 2, although in a few instances, the rockets were nearly superimposed (see UF92 and UF524 in Figure 18). Also, the type 1 and type 2 precipitin bands of each strain showed a reaction of non-identity, thereby extending to A. viscosus and A. naeslundii strains in general the observation, initially made on the basis of studies of T14V fimbriae (14,92), that type 1 and type 2 fimbriae are immunologically distinct entities.

However, there are shared determinants among fimbrial populations of the same type as demonstrated by the reaction of R10 or R59 with the type 1 fimbriae of A. naeslundii serotypes 2 and 3 and A. viscosus serotype 2. Likewise, R2P and R55 reacted with the type 2 fimbriae of heterologous species. The fact that R59 against T14V type 1 fimbriae reacted strongly with the type 1 fimbriae of all 10 strains of A. naeslundii serotype 3, whereas R55 and R70 against the type 2 fimbriae of T14V and WVU45, respectively, reacted with only a few strains suggests that the type 1 fimbriae of different strains are more closely related antigenically than are the type 2 fimbriae.

These observations will be discussed more fully after presentation of the results of the bacterial agglutination assays.

Bacterial Agglutination

The monoclonal and polyclonal antibodies specific for N16 fimbriae were evaluated by agglutination versus cells of different species and serotypes to assess the incidence of shared fimbrial epitopes among heterologous isolates. Although this had been done to a very limited extent by XIEP-A using fimbrial antigens that had been extracted with hot acid, bacterial agglutination assays permitted a more extensive survey and also enabled the evaluation of antigenic relatedness among fimbriae in their native configuration.

Rabbit anti-N16 type 1 fimbriae, rabbit anti-N16 type 2 fimbriae, and two MAbs reactive with N16 fimbriae were tested by bacterial agglutination against a total of 106 isolates of various oral bacteria. The panel of isolates included representatives of the following species: Actinomyces bovis, A. israelii, A. naeslundii, A. odontolyticus, A. pyogenes, A. viscosus, A. meyeri, Arachnia propionica, Bacteroides loescheii, Rothia dentocariosa, Streptococcus mutans, S. salivarius, S. cricetus, S. sobrinus, S. mitis, S. rattus, and S. sanguis. Only isolates of A. naeslundii and A. viscosus were agglutinated by any of these anti-fimbrial antibodies. Since fimbriae are present on many of the other species, for example A. bovis (73), A. israelii (26,73), B. loescheii (105), and S. sanguis (41), the inability of antibodies against N16 fimbriae to agglutinate them suggests that their fimbriae are not antigenically related to N16 fimbriae.

The bacterial agglutination patterns of selected isolates representative of various serotypes of A. naeslundii and A. viscosus are shown in Table 7.

R10 against N16 type 1 fimbriae agglutinated A. naeslundii serotypes 2 and 3, Fillery cluster 3 (42), and A. viscosus serotype 2 strains, but it did not agglutinate strains of A. naeslundii serotypes 1 and 4. Clark et al. (28) obtained the same reaction pattern using RIgG against T14V type 1 fimbriae in an immunodot EIA. R2P against N16 type 2 fimbriae agglutinated both serotypes of A. viscosus and all A. naeslundii isolates except serotypes 2 and 4. MAb 3B5.A1 agglutinated only the A. naeslundii serotype 3 isolates and one of the Fillery cluster 3 isolates, whereas MAb 2B5.B6 agglutinated less than 50% of the A. naeslundii serotype 3 strains plus several strains that are difficult to serotype. The agglutination results, for the most part, were consistent with the results obtained by RIA and XIEP-A.

Although N16 type 1 fimbriae are unrelated antigenically to type 2 fimbriae, as demonstrated by immunodiffusion and XIEP-A, the results of both XIEP-A and bacterial agglutination indicate that within a fimbrial population (type 1 or type 2), there are antigenic determinants that are common to other serotypes and species, as well as determinants that are unique to certain serotypes or other subsets of a species, as first suggested by the agglutination reaction patterns of MAbs against T14V type 2 fimbriae (14).

Other investigators have used many of the strains listed in Table 7 to study the functional and antigenic relationships among Actinomyces fimbriae. Some of their findings, which have been based almost exclusively on the use of Abs specific for T14V or WVU45

TABLE 7. Bacterial agglutination patterns of selected isolates

Antigens	Antibodies[a] specific for N16 fimbriae			
	R10	R2P	3B5.A1	2B5.B6
A. naeslundii serotype 1				
W826 (WVU45)	0[b]	320	0	0
X569, W734, W869 (WVU398A)	0	10	0	0
A. naeslundii serotype 2				
W752	10	0	0	0
W1544	20	0	0	0
A. naeslundii serotype 3				
N16 (WVU820)	10	160	≥20480	640
WVU1267	40	5120	≥20480	0
WVU1468	20	640	≥20480	0
WVU1527	40	640	≥20480	2560
WVU1528	20	320	≥20480	1280
W1629	10	160	≥20480	0
W2273	80	5120	≥20480	0
W2821	80	5120	≥20480	10240
UF92	80	≥20480	≥20480	0
UF524	160	2560	≥20480	0
A. naeslundii Fillery cluster 3				
B74	20	80	0	0
B120, RIK21	40	160	0	0
H3-3-8	40	5120	≥20480	0
RIK149	20	320	0	0
A. naeslundii serotype 4				
WVU924, WVU963	0	0	0	0
A. naeslundii untypables				
X600	10	1280	0	640
W735	0	1280	0	640
WVU911	0	640	0	640
UF76	0	2560	0	1280
PK29	40	1280	0	2560
WVU903	160	2560	0	10240
WVU825	0	80	0	0
W1911	160	320	0	0
E38W3	20	80	0	0
W1716	0	640	0	0
A. viscosus serotype 1				
M482(T6), M484, X602, A755	0[c]	10	0	0
A757, A758, A759	0	20	0	0
A. viscosus serotype 2				
T14V	40	320	0	0
T14AV	640	1280	0	0
W859	160	320	0	0
W863, W1628	0	80	0	0
W872	40	10	0	0
M100	10	320	0	0
W1053	10	40	0	0

Table 7--continued

[a] R10 = rabbit antiserum against N16 type 1 fimbriae
R2P = rabbit antiserum against N16 type 2 fimbriae
3B5.A1 = MAb 3B5.A1 ascites against N16 type 2 fimbriae
2B5.B6 = MAb 2B5.B6 ascites against N16 type 2 fimbriae

[b] If the cells did not agglutinate at a 1:10 dilution of antibody, the reaction was recorded as 0. Positive reactions were expressed as the reciprocal of the highest dilution of antibody that gave complete agglutination.

[c] Although all A. viscosus serotype 1 isolates were negative at a 1:10 dilution of R10 antiserum, all were positive at dilutions ranging from 1:2 to 1:32 when tested against protein A - purified RIgG at 1 mg/ml.

fimbriae, will be discussed in relation to the results obtained in this study with Abs specific for the fimbriae of N16.

In the original classification scheme of Fillery et al. (42), based on numerical taxonomy and serology, all human isolates of A. viscosus and A. naeslundii were assigned to one of six clusters. Since the A. viscosus clusters (1, 2, 4, and 6) could not be easily differentiated even with antifibril antisera (76), all A. viscosus isolates now are generally assigned to cluster 1 and A. naeslundii isolates to cluster 3 or cluster 5 (38), a separation that has been supported by DNA hybridizations (30). The relationships between cluster assignment and serotype (46) will be explained in the following discussion.

A. naeslundii serotype 1, often referred to as cluster 5 or "typical" A. naeslundii, has only one type of fimbriae, type 2 fimbriae. In a study of the type strain (WVU45 = W826 = ATCC 12104) and a spontaneously occurring mutant, WVU45M, Cisar et al. (17) observed that WVU45 was agglutinated by RIgG specific for T14V type 2 fimbriae but not with RIgG specific for T14V type 1 fimbriae and that R70 RIgG specific for WVU45 fimbriae agglutinated T14V but not PK455, a mutant of T14V that lacks type 2 fimbriae. Also, the mutant (WVU45M) did not agglutinate with R70 or with RIgG specific for T14V type 1 or type 2 fimbriae, and it did not have fimbriae when examined by immunoelectron microscopy. The agglutination of A. naeslundii serotype 1 strains by R2P against N16 type 2 fimbriae but not by R10 against N16 type 1 fimbriae is consistent with their conclusion that these strains possess only type 2 fimbriae.

In the same study Cisar et al. reported that strain W1544 agglutinated with RIgG specific for either type of T14V fimbriae, which suggested that W1544 had both types of fimbriae. The existence of a second serotype of A. naeslundii was first reported in 1972 (4), and W1544 was designated as a typical representative of A. naeslundii serotype 2 (5). These strains appeared to be catalase-negative variants of A. viscosus serotype 2 (personal observation). Evidence in support of this hypothesis was provided by numerical taxonomy (38,42) and indirect immunofluorescence with antifibril antisera (38) using strain W752; also, Coykendall and Munzenmaier (30) showed that by S_1 nuclease DNA hybridization W752 had 98% homology with WVU627, a cluster 1 or "typical" A. viscosus serotype 2 isolate, and only 46% and 33% homology with cluster 3 and cluster 5, respectively. On the basis of these studies, A. naeslundii serotype 2 strain W752 was assigned to cluster 1 with all the A. viscosus serotype 2 isolates. Clark et al. (25) showed that W752 and W1544 adsorbed as well to SHA as most A. viscosus serotype 2 strains, which suggests that these strains have type 1 fimbriae and, therefore, are more similar to typical A. viscosus than to typical A. naeslundii in that regard. W1544 and W752 also have been shown to exhibit the lectin activity associated with type 2 fimbriae (29,37). Thus, on the basis of both functional and antigenic characteristics it is clear that these strains have both types of fimbriae. In view of the extremely close similarity between A. naeslundii serotype 2 and A. viscosus serotype 2 strains, it is interesting to note that W752 and W1544 did not react with R2P antiserum, even though R2P agglutinated all of the A. viscosus serotype 2 strains tested.

However, the most interesting results were those obtained with the A. naeslundii serotype 3 and Fillery cluster 3 isolates. These two groups are generally considered to be equivalent (25,38). B74 and B120 were among the original isolates assigned to cluster 3 by Fillery et al. (42). In that study these "atypical" A. naeslundii strains fell in between the typical A. viscosus isolates (cluster 1) and the typical A. naeslundii isolates (cluster 5) on a similarity matrix. Like typical A. naeslundii, cluster 3 strains were catalase-negative; like typical A. viscosus, they had galactose rather than galactosamine in their cell wells, and they completely hydrolyzed aesculin. Like W752 (A. naeslundii serotype 2) but unlike any of the other cluster 1 isolates or cluster 5 isolates, cluster 3 isolates had electrophoretically fast moving 6-phosphogluconate dehydrogenase. It is unfortunate that their study did not include N16 or any of the other A. naeslundii serotype 3 strains (46,57) for a direct comparison to cluster 3 isolates. Even in the DNA hybridization study of Coykendall and Munzenmaier (30), which did include WVU820 (N16) and B74, the two strains were evaluated separately by different methods.

Clark et al. (23,25,28) have demonstrated both similarities and differences between A. naeslundii serotype 3 and cluster 3 strains. The relative hydrophobicity of N16 or WVU1468 was lower than that of each of 5 cluster 3 isolates (B74, B120, RI-K47, RI-K21, and H3-3-8), but all the strains adsorbed equally well to SHA (25). Even though all the isolates were positive by immunodot EIA versus RIgG to T14V type 1 fimbriae (28), they were not affected to the same degree when RIgG against T14V type 1 fimbriae was used to inhibit adsorption of

the cells to SHA; for example, WVU1468 was inhibited 91% but H3-3-8 was not inhibited at all (23).

The agglutination of all 5 of the cluster 3 isolates by the polyclonal antiserums against N16 type 1 and type 2 fimbriae, as shown in Table 7, is evidence of antigenic relatedness between cluster 3 and serotype 3 strains. On the other hand, since the 3B5.A1 epitope appears to be an important antigenic determinant for the A. naeslundii serotype 3 isolates, its absence on 4 of the 5 cluster 3 strains, including B74, suggests that there are at least two serogroups in cluster 3, one with the 3B5.A1 epitope and the other without it. The absence of the 3B5.A1 epitope on B74 fimbriae might explain why antiserum to B74 fibrils, after adsorption with A. viscosus cells, reacted with slightly less than 50% of cluster 3 isolates by indirect immunofluorescence (38).

The strains originally identified by Gerencser and Slack (46) as A. naeslundii serotype 4 do not appear to belong to this species. In this study the two strains examined were not antigenically related to N16 fimbriae as demonstrated by bacterial agglutination, and WVU924 did not react in XIEP-A with any of the Abs against fimbriae of N16, WVU45, or T14V. Also, Clark et al. have shown that WVU963 did not absorb to SHA (25) or react with RIgG against T14V type 1 fimbriae (28). However, these strains make up a serologically identifiable group (46), and similar strains are often isolated from the oral cavity, although now they are usually referred to as just Actinomyces serotype 963 without any species designation (87).

The group of isolates designated A. naeslundii untypables includes strains that resemble A. naeslundii physiologically and

biochemically but whose reactions with the fluorescent antibody reagents used for serotyping do not allow unequivocable placement into one serotype or another. The agglutination of many of the untypables with MAb 2B5.B6, a MAb that reacts with less than 50% of the isolates of the homologous serotype, points out the potential difficulties of serotyping with polyclonal reagents. Also, the agglutination of W735, WVU911, and UF76 with MAb 2B5.B6 and R2P but not with R10 suggests that the 2B5.B6 epitope is on the type 2 fimbriae, an observation that was confirmed by its reactivity with type 2 fimbrial subunits on immunoblots. Gerencser and Slack (46) had suggested that WVU903 and WVU825 represented different serotypes within serotype 1. Although it is possible that WVU825 belongs in that serotype because it appears to lack type 1 fimbriae like other isolates of that group, clearly WVU903 cannot be considered to be a typical serotype 1 isolate. Its agglutination with R10 suggests that it has type 1 fimbriae, and Clark et al. have shown that it adsorbs well to SHA (25).

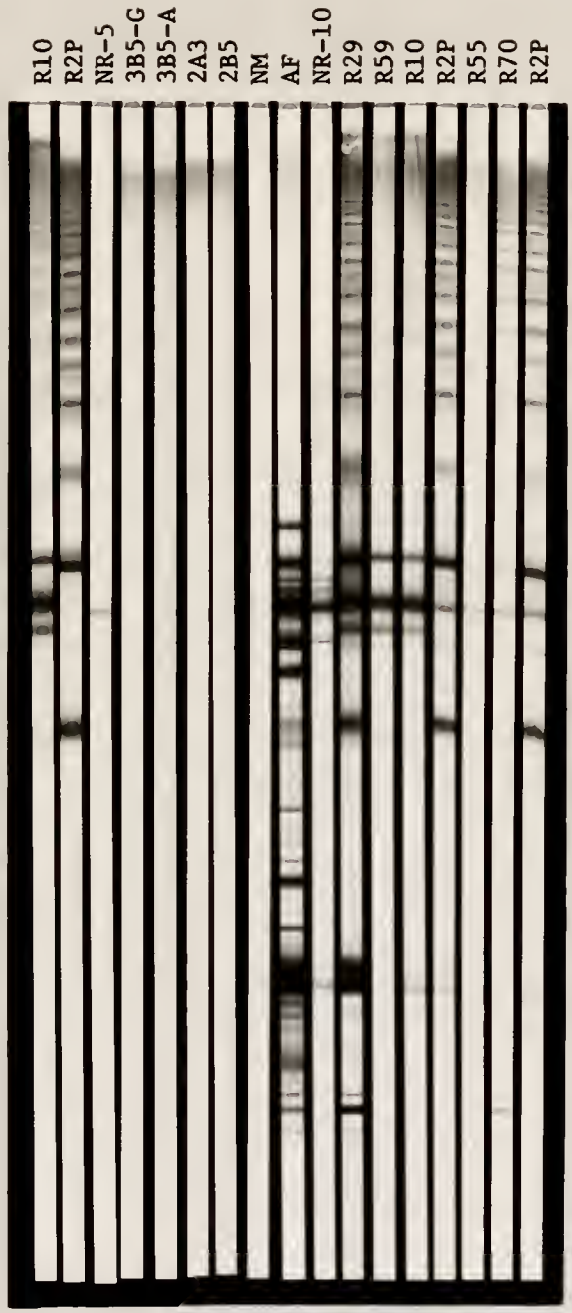
In summary, the agglutination of A. viscosus isolates by polyclonal antiserums R10 and R2P against A. naeslundii N16 fimbriae suggests that the fimbriae of these two species possess common epitopes, but the reactions of MAbs 3B5.A1 and 2B5.B6 indicate that certain fimbrial epitopes are unique to a species or serotype or other subgroup within a species. The presence of both common and unique epitopes on the same structure or molecule provides an explanation for the difficulty in obtaining serotype-specific or even species-specific polyclonal reagents and suggests that the use of a MAb or a panel of MAbs with the desired specificities would be a better approach.

Immunoblot Analysis

Blot strips of SDS-PAGE resolved proteins from N16 crude French press supernatant were developed with various monoclonal and polyclonal antibodies specific for fimbriae of N16, T14V, or WVU45; the results for reactions with DTT-reduced antigen are shown in Figure 21. All antibodies specific for fimbriae reacted with the ladderlike series of high molecular weight bands, whereas normal sera did not react with these bands. As for the reactions with fimbrial subunits, all 3 MAbs against N16 fimbriae reacted with the type 2 band at 63 kd but not with the 39-40 kd doublet. When the MAbs were tested against immunoblots of unreduced N16 antigens (data not shown), they all reacted with the high molecular weight bands and with the 62-63 kd doublet seen in unreduced type 2 fimbriae but not with any lower molecular weight bands. The failure of the MAbs to react with the 39-40 kd doublet may indicate that those are not fimbrial bands or that those bands lack the appropriate epitopes. Evidence in support of the latter explanation comes from the observation that polyclonal R2P and R70 against N16 and WVU45 type 2 fimbriae, respectively, reacted with the 39-40 kd doublet, whereas R55 against T14V type 2 fimbriae did not.

R59 against T14V type 1 fimbriae gave virtually the same pattern of reactivity as R10, confirming the antigenic relatedness between the T14V and N16 type 1 fimbriae as determined by XIEP-A. R55 against T14V type 2 fimbriae and R70 against WVU45 type 2 fimbriae reacted with N16 type 2 fimbrial bands by immunoblot even though XIEP-A had indicated little or no relationship.

Figure 21. Demonstration of the specificities of various monoclonal and polyclonal antibodies for A. naeslundii N16 crude French press supernatant antigens by immunoblot analysis. The antigen was reduced with DTT at 100 C. The specificities of these antibodies have been defined: R10 and R2P = RIgG at 5 µg/ml; NR-5 and NR-10 = normal RIgG at 5 and 10 µg/ml, respectively; 3B5-G and 3B5-A = MAb 3B5.A1 IgG at 10 µg/ml and ascites at 1:100, respectively; 2A3 = MAb 2A3.B3 culture supernatant; 2B5 = MAb 2B5.B6 ascites at 1:100; NM = normal mouse ascites at 1:100; AF = Aurodye forte total protein stain; R29 = antiserum at 1:1500; R59, R55, and R70 = RIgG at 10 µg/ml.


R10
R2P
NR-5
3B5-G
3B5-A
2A3
2B5
NM
AF
NR-10
R29
R59
R10
R2P
R55
R70
R2P

In summary, the results of immunoblot analysis of the reactivity of various fimbriae-specific antibodies with N16 antigens confirms the reported specificities of these antibodies for type 1 or type 2 fimbriae and supports the proposed patterns for N16 fimbrial subunits as seen in Figure 14.

SUMMARY AND CONCLUSIONS

The original goals of this study were to (a) produce monoclonal antibodies against surface antigens of A. naeslundii serotype 3 strain N16, (b) select at least one MAb that exhibited serotype specificity, and (c) use the MAb as a tool to isolate and characterize the surface component carrying the serotype-specific epitope. After it was determined that the serotype-specific epitope was located on the fimbriae, the following additional goals were set: (d) determine which type of fimbriae carried the epitope, (e) produce polyclonal antibodies against that fimbrial type or both fimbrial types, and (f) use the polyclonal and monoclonal antibodies in a variety of immunological assays to examine the antigenic relatedness between the fimbriae of A. naeslundii N16 and the fimbriae of heterologous strains.

Three MAbs that bound to N16 cells in a solid-phase RIA were produced; these were MAb 3B5.A1 (IgG_{2a}), MAb 2A3.B3 (IgG_3), and MAb 2B5.B6 (IgM). When they were tested by RIA against a panel of cells that included 10 A. naeslundii serotype 3 strains and 51 heterologous strains, MAb 3B5.A1 was the only one that reacted with all of the homologous and none of the heterologous isolates. Thus, it was the only MAb that appeared to be serotype-specific. MAb 2A3.B3 cross-reacted with other serotypes of A. naeslundii and with A. viscosus serotype 2, whereas MAb 2B5.B6 reacted with only 40% of the homologous isolates plus a few other A. naeslundii strains. Electron microscopy of N16 cells indirectly immunolabeled with

colloidal gold indicated that all three MAbs bound to epitopes located on fimbriae.

There was both immunological and functional evidence from the work of other investigators to suggest that N16 had both type 1 and type 2 fimbriae. In the present study, all of the A. naeslundii serotype 3 strains were shown to have type 1 fimbriae by their cross-reactions with rabbit IgG specific for A. viscosus T14V type 1 fimbriae. In this respect, serotype 3 strains differ from A. naeslundii serotype 1 strains, which do not have type 1 fimbriae (17). All of the serotype 3 strains were shown also to have type 2 fimbriae by their ability to cause lactose-inhibitable hemagglutination. Since the type 2 fimbriae mediate hemagglutination by Actinomyces cells, the removal of type 2 fimbriae from the cells was monitored by measuring the decline in the HA titer of the treated cells relative to untreated cells.

In preliminary experiments fimbriae were extracted from N16 cells by several different methods. French press shearing and sonication were found to remove fimbriae from cells much more effectively than Virtis homogenization or lithium chloride extraction. Lancefield extraction was a convenient way to prepare soluble antigens for Laurell or crossed rocket immunoelectrophoresis, but it extracted too much carbohydrate to be considered as a means for obtaining fimbriae for purification.

Type 1 and type 2 fimbriae were detected in Lancefield extracts and in crude sonicates or French press supernatants by crossed immunoelectrophoresis using rabbit antiserum against N16 whole cells. When ^{125}I-MAb 3B5.A1 was also incorporated into the gel,

autoradiography indicated that MAb 3B5.A1 was specific for the type 2 fimbriae. MAb 3B5.A1 proved to be a valuable tool for purifying the type 2 fimbriae of A. naeslundii N16. When crude sonicates of N16 cells were chromatographed on Bio-Gel A-5m columns, the fractions containing type 2 fimbriae were identified by their positive reactions with MAb 3B5.A1 in an immunodot EIA. The type 2 fimbriae were then purified from these fractions by chromatography on a MAb 3B5.A1 immunoaffinity column. The type 2 fimbriae recovered from the MAb column were slightly contaminated with type 1 fimbriae, probably as a result of noncovalent interactions between the two types of fimbriae or nonspecific adsorption to the column; this might have been avoided by inclusion of a detergent, such as 0.05% Tween 20, in the buffer for eluting the unbound fraction, as suggested by Cisar et al. (17).

Polyclonal antisera monospecific for N16 type 1 or type 2 fimbriae were produced by immunizing rabbits with fimbrial immunoprecipitins excised from XIEP gels. Immunoaffinity columns prepared from these antisera required much harsher elution conditions than the MAb column in order to desorb bound antigens; consequently, rabbit IgG leached from the columns, contaminating the fimbrial samples.

The effects of various physical and chemical treatments on N16 fimbriae were assessed by immunoblot analysis of SDS-PAGE resolved antigens. Fimbrial bands were not detectable in samples treated with Pronase, proteinase K, or papain. Base and hot acid also altered the fimbrial immunoblot patterns significantly. N16 fimbriae could not be completely dissociated, but there was evidence for partial dissociation into subunits. Type 1 and type 2 fimbriae could be

distinguished by their different subunit patterns on immunoblots, and both were affected by increased temperature and by reduction. N16 fimbriae appear to have heat modifiable subunits like those reported for T14V type 1 fimbriae and WVU45 type 2 fimbriae, but that would have to be verified with purified or cloned subunit proteins as Yeung et al. (108) have done. The fimbrial patterns on immunoblots of crude N16 fimbriae are so complex that it is not possible to tell precisely what shifts in molecular weight are occurring. When samples were reduced at 100°C, N16 type 1 fimbrial subunits were observed at 65 kd, 57-60 kd, and a weaker doublet at 53-54 kd, and type 2 subunits occurred at 63 kd with a doublet also at 39-40 kd in some samples.

Antibodies specific for the fimbriae of N16 (A. naeslundii serotype 3), T14V (A. viscosus serotype 2), and WVU45 (A. naeslundii serotype 1) were used in several different immunological assays, including Ouchterlony analysis, crossed immunoelectrophoresis with autoradiography, bacterial agglutination, and SDS-PAGE immunoblot, to examine the antigenic relationships among Actinomyces fimbriae. The results of these assays indicated that (a) N16 type 1 fimbriae are not related antigenically to the type 2 fimbriae, (b) the type 1 fimbriae of A. naeslundii serotypes 2 and 3 and A. viscosus serotype 2 have common antigenic determinants, (c) type 2 fimbriae of certain A. naeslundii and A. viscosus strains also have common antigenic determinants, and (d) MAb 3B5.A1 recognizes a serotype-specific determinant located on the type 2 fimbriae of A. naeslundii serotype 3 strains.

One of the more interesting findings was that MAb 3B5.A1 did not agglutinate 4 of the 5 strains belonging to Fillery cluster 3. Since

A. naeslundii serotype 3 and Fillery cluster 3 are generally considered to be equivalent (25,38), this data suggests that there are at least two serogroups in cluster 3, one with the 3B5.A1 epitope and the other without it. Also, the absence of the 3B5.A1 epitope on B74 could explain why antiserum to B74 fibrils, after adsorption with A. viscosus cells, reacted with less than 50% of the cluster 3 isolates by indirect immunofluorescence (38). If a strain possessing the 3B5.A1 epitope, for example cluster 3 strain H3-3-8 or any of the A. naeslundii serotype 3 strains, were used to make cluster 3 specific antifibril antisera, it would probably react with a higher percentage of the cluster 3 isolates than B74 antiserum.

This study was primarily an immunological characterization of the fimbriae of A. naeslundii N16 and was somewhat weighted toward the type 2 fimbriae because all the MAbs reacted with type 2 fimbriae, in particular MAb 3B5.A1, which recognized a serotype-specific epitope. Consequently, there is plenty of room for additional studies, particularly in regard to the functional and immunological properties of the type 1 fimbriae and functional properties of type 2 fimbriae.

The difficulties of purifying one type of fimbriae when both are present and the difficulties of preparing polyclonal antisera monospecific for one fimbrial type have been discussed previously (20). Although the production of MAbs can overcome the latter problem, polyclonal antibodies are more useful in some instances. The availablility of mutants lacking one or both fimbriae can facilitate purification of fimbriae, production of monospecific antisera, as well as attribution of functional properties to one type or the other (17,67,68,84); Cisar et al. (16) have already shown that loss of one

fimbrial type did not alter expression of the other. Thus, any additional studies with N16 should probably start with the isolation of 1^+2^-, 1^-2^+, and 1^-2^- mutants. Since fimbriae-specific antibodies are already available, mutants could be isolated by the colony blotting technique of Reis et al. (91); mutants of other strains have been isolated successfully by enrichment techniques (17,67). Since one of the N16 cell lines was observed to be a mix of about 80% 1^+2^- and 20% 1^+2^+ (data not presented), isolation of a coaggregation-defective (1^+2^-) mutant of N16 should be relatively easy.

It would also be helpful to have large panels of MAbs specific for different type 1 and type 2 fimbrial epitopes. These could be useful typing reagents, but more importantly, the availability of many different MAbs would increase the likelihood of finding some that could inhibit the functional activities of the fimbriae. Even some polyclonal antisera that bind to a fimbrial type cannot inhibit its functional activity (28), so the chances of finding a MAb that would do so would be even slimmer.

Fab fragments of both polyclonal and monoclonal antibodies should be tested for their ability to inhibit functional activities; for example, MAb 3B5.A1 should be tested for its ability to inhibit coaggregation, whereas antibodies to type 1 fimbriae could be assayed for their ability to inhibit adsorption to SHA (23) or to proline-rich protein (PRP)-coated hydroxyapatite beads (48). These antibodies could be used to screen recombinants for expression of N16 fimbrial genes encoding for functional domains; MAb 3B5.A1 could be used to detect recombinants expressing the N16 type 2 fimbrial gene encoding

for the serotype-specific epitope. After subcloning to obtain the smallest DNA fragment encoding for the desired fimbrial domain, the cloned protein could be purified, using MAbs as a tool to monitor purification or for immunoaffinity chromatography.

The nature of the cloned protein would determine the next step. If it was the serotype-specific epitope, the protein could be tested for its ability to inhibit binding of MAb 3B5.A1 to N16 cells. If it was the binding domain of the lectin or the domain for adsorption to SHA, the cloned protein would be tested as an inhibitor of coaggregation or adsorption to SHA, respectively. Fimbrial subunits could be cloned and the structural basis for heat modifiability investigated, as Yeung et al. (108) have done for the 65 kd T14V type 1 subunit.

The amino acid sequence of the cloned protein and the DNA sequence of the corresponding gene could be determined. The cloned protein or synthetic peptides could be tested as inhibitors of the appropriate binding activity or used as immunogens to elicit antibodies that could inhibit binding. Intact fimbriae of T14V have been tried as vaccines in an experimental animal model, and there was a reduction in colonization of immunized animals relative to control animals (23). However, even though Actinomyces fimbriae appear to have potential as vaccines, it is unlikely that a vaccine containing a single strain would be effective against all strains because the antigenic determinants of one strain may not be near the site that mediates adsorption to SHA in other strains (23). It is even more unlikely that a synthetic peptide or cloned protein subunit would be

an effective, broad-spectrum vaccine. However, cloning the fimbrial genes will give a clearer picture of the molecular basis for adherence in Actinomyces and perhaps suggest other strategies for interfering with the pathogenic mechanisms.

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BIOGRAPHICAL SKETCH

Sandra L. Bragg was born June 16, 1947 in DeLand, Florida. She attended elementary school in Kirksville, Missouri, while her father completed his medical education. In 1955 her family moved to Daytona Beach, Florida, where she participated in competitive tennis. After graduating from Seabreeze Senior High School in 1965, Sandra moved to Atlanta, Georgia. In 1969, she received her Bachelor of Arts degree with a major in religion from Emory University. She then completed her Master of Science degree in biology at Georgia State University in 1972 and has since been employed as a microbiologist in the Division of Mycotic Diseases at the Centers for Disease Control (CDC). She was awarded a CDC scholarship to pursue the Doctor of Philosophy degree and moved to Gainesville for two years in 1978 to complete her course work. She then returned to CDC in 1980 and has worked on both her dissertation research and CDC research projects in mycology since that time.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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